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Declarations of interest: none

Running title: BH4 and neuroinflammation

Abbreviations: Arg, BH2, BH4, DA, DHFR, DOPAC, GCH1, GFRP, GTP, HVA, IL1β, IL6, IL10, IFNα, LPS, MCP-1, NO, NOS, Phe, PAH, PTPS, SR, TH, TNFα, Tyr

Key words: BH4, Dopamine, Inflammation, Depression

Abstract

Tetrahydrobiopterin (BH4) is a necessary cofactor for the synthesis of monoamines from essential amino-acids, phenylalanine, tyrosine and tryptophan. The BH4 synthesis pathway is induced by inflammatory factors but highly regulated processes maintain levels in a physiological range. However, BH4 activity can be durably altered in inflammation-related pathologies, such as certain types of depression, potentially involving impairment of dopaminergic neurotransmission. The purpose of this study was to investigate the response of the brain BH4 pathway to the inflammatory stimulus induced by lipopolysaccharide (LPS) in mice. Brain expression of genes related to BH4 synthesis, levels of BH4, changes in L-aromatic amino acid precursors of monoamines and dopamine levels were determined. As secondary aim, the effect of acute BH4 supply under the inflammatory challenge was tested on these parameters and on the expression of inflammatory cytokines. Mice were also submitted to the sucrose preference test and to the open-field in order to asses hedonic and locomotor responses to LPS, in addition to their modulation by BH4 supply. The LPS challenge resulted in decreased striatal DA levels and increased Phenylalanine/Tyrosine ratio, suggesting reduced BH4 activity. BH4 supply was effective to increase striatal BH4 levels, to restore the LPS-induced decreased in DA levels in striatum and to dampen the LPS-induced expression of inflammatory cytokines. At the behavioral level, BH4 supply was able to restore the loss of locomotor response to amphetamine in the LPS treated mice, suggesting a modulation of the dopaminergic neurotransmission.

These data suggest that BH4 can be considered as a potential add-on molecule, helping to maintain or restore dopaminergic neurotransmission in neuroinflammatory conditions.

Introduction

Tetrahydrobiopterin (BH4) is an obligatory cofactor for aromatic amino acid hydroxylases, converting the amino acid phenylalanine (Phe) into tyrosine (Tyr), which in turn is converted to L-DOPA by tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine (DA) synthesis (Werner et al. 2011). BH4 is also necessary for the function of nitric oxide synthases (NOS), with a greatest affinity for production of nitric oxide (NO) (Tayeh and Marletta 1989). BH4 is therefore a key player in the synthesis and homeostasis of monoaminergic neurotransmitters, and changes in its availability can be associated to alterations in monoaminergic neurotransmission (Fanet et al. 2021, Vancassel et al. 2018). Both at the periphery and centrally, the *de novo* synthesis of BH4 occurs from the precursor guanosine triphosphate (GTP) by the concert action of the GTP cyclohydrolase 1 (GCH1), 6-pyruvoyltetrahydrobiopterin synthase (PTPS) and sepiapterin reductase (SR) (Thöny et al. 2000 (**Fig. 1A**). Once synthetized, BH4 is easily oxidized to the inactive BH2, which in turn can be converted back into BH4 throughout the alternative salvage pathway involving action of dihydrofolate reductase (DHFR). DHFR is important not only for the synthesis of BH4 but also for the maintenance of homeostatic levels of BH4/BH2 (Harada et al. 1993, Werner et al. 2011). Moreover, activity of GCH1 is regulated through interaction with GFRP (GTP Feedback Regulatory Protein), mediating BH4 feedback inhibition and Phe feed-forward stimulation of GCH1 activity (Neurauter et al. 2008). The expression and activity of GCH1 is massively increased under a pro-inflammatory stimulus (Kaneko et al. 2003, Werner et al. 1993, Werner-Felmayer et al. 1993), a scenario that leads to reduced bioavailability of BH4 due to its high autoxidation and its preferential use by NOS (Fanet et al. 2021). As a consequence, the depletion of BH4 can compromise the activity of strictly dependent enzymes which ensure the production of monoamines (especially DA) that, in turn, will induce behavioral symptoms related to altered dopaminergic neurotransmission (Fanet et al. 2021, Vancassel et al. 2018).

Clinical data from patients with juvenile arthritis show that the status of chronic inflammation is associated to decreased BH4 activity, measured through an increased Phe/Tyr ratio (Korte-Bouws et al. 2019). Data from pro-inflammatory cytokine therapies (interferon, IFN-α) for chronic viral diseases and cancer, showed associations between increased levels of inflammatory markers and decreased BH4 in the cerebrospinal fluid (Felger 2019, Felger and Lotrich 2013, Miller et al. 2017, Zoller et al. 2012). Moreover, treatment with IFN-α has been shown to increase the Phe/Tyr ratio and reduce BH4 activity, which was correlated with fatigue, anhedonia, motor slowing and reduced cerebrospinal fluid levels of DA (Capuron et al. 2007, Capuron and Miller 2004, Felger and Treadway 2017, Neurauter et al. 2008). In addition, neuroimaging studies showed that basal ganglia are specifically targeted by pro-inflammatory cytokines, inducing alterations in presynaptic DA levels and turnover (Capuron et al. 2002, 2012, Eisenberg et al. 2010, Felger and Lotrich 2013, Miller et al. 2013). This clinical evidence parallels data obtained in non-human primates showing that reduced motivation arising in response to IFN-α is accompanied by a reduction in striatal DA metabolism (Felger et al. 2013). This corroborates recent findings from gene signature analyses in peripheral blood immune cells that confirm low Tyr metabolism in depressed patients with inflammatory profile and high anhedonia (Bekhbat et al. 2020). It has also been shown that fatigue and neurovegetative symptoms are less responsive to conventional antidepressant therapies (Felger and Lotrich 2013, Strawbridge et al. 2015), suggesting the involvement of distinct neurobiological mechanisms in cytokine-induced depressive phenotypes that remains to be fully understood.

Consistent with this, reduced BH4 levels have been reported in *postmortem* brain of depressed subjects (Blair et al. 1984, Fanet et al. 2021) whereas administration of BH4 induced some improvement in depressive symptoms in small clinical assays (Curtius et al. 1983, Pan et al. 2011).

Taken together, these data suggest that inflammation alters DA function in basal ganglia, possibly through changes in BH4 bioavailability, and this can contribute to the onset or development of depressive symptoms. However, the effects of BH4 administration on inflammation-induced neurobehavioral disturbances remain largely unknown and a more detailed understanding of the role of the BH4 pathway in these processes is necessary.

For this purpose, we used the model of acute systemic administration of the cytokine inducer lipopolysaccharide (LPS) in mice to explore the involvement of the BH4 pathway in neurobiological and behavioral inflammatory responses, including changes in amino acid precursor of DA synthesis and in the expression of enzymes/proteins responsible for BH4 production. The effects of LPS were measured 24h after administration, when LPS-induced sickness behavior is minimal, while LPS-induced depressive-like behavior persists (Frenois et al. 2007, O'Connor et al. 2009). Our aim was also to assess the impact of BH4 administration under inflammatory condition on the neurobiological correlates of the behavioral alterations reported 24h after LPS challenge. We previously showed that levels of BH4 significantly increased in the brain 3h after an acute administration of BH4 at a dose of 50 mg/kg (Fanet et al. 2020). Thus, we tested the ability of BH4 administration to modulate local responses to the immune challenge 21h after LPS, through the measure of pro-inflammatory cytokines, BH4 pathway activity and DA metabolism. Locomotion and hedonic response of mice were also assessed as behavioral outcomes.

Materials and Methods

Animals and treatments

Male C57BL/6J mice aged 8 weeks were purchased from Janvier Labs (Le Genest-Saint-Isle, France). They were housed in groups of 5 animals in standard polypropylene cages and maintained in a temperature- and humidity-controlled facility under a 12:12 LD cycle (8:00 on), with *ad libitum* consumption of water and food (Standard Rodent Diet A04, SAFE, Augy, France). All animal care and experimental procedures followed ethical protocols, in accordance with the EU Directive 2010/63/EU for animal experiments. They were approved by the Region Aquitaine Veterinary Services (Direction Départementale de la Protection des Animaux, approval ID: A33-063-920) and by the ethics committee of animal experimentation of Bordeaux (CE_{050}). Every effort was made to minimize suffering and the number of animals used.

Mice were free to acclimate to their new environment for two weeks before behavioral testing began. On the day of test, mice received an intraperitoneal (i.p.) injection of either sterile saline solution (NaCl 0.9%; Controls) or LPS at the dose of 830 μg/kg (*E.coli*, serotype 0127:B8, Sigma Aldrich, Lyon, France), in order to induce a transient neuroinflammation and a distinct depressive-like behavioral phenotype (Frenois et al. 2007 ; O'Connor et al. 2009). BH4 stock solution was prepared by dissolving 1 g of (6R,S)-5,6,7,8-Tetrahydro-L-biopterin dihydrochloride (Schircks laboratories, Switzerland) in a mix solution of 10 mL of 0.1 M HCl + 0.02% dithiotreithol (DTT) and stored protected from light at -80°C until use. Twenty-one hours after LPS injection, mice were injected i.p. with saline (NaCl 0.9% + 5% HCl 0.1M) or BH4 solution diluted in NaCl 0.9%, at the dose of 50 mg/kg, **immediately**

before use (Fanet et al. 2020). Then four experimental groups were constituted: NaCl, LPS, NaCl+BH4, LPS+BH4). The experimental design is summarized in **Fig. 1B**.

Amphetamine challenge

Locomotor activity was measured in the four experimental groups in basal conditions and in response to an amphetamine (AMPH) challenge. D-Amphetamine (Merck, France) was dissolved in saline and injected at the dose of 3 mg/kg i.p., in a volume of 10 μL/g (Fanet et al. 2020). Corresponding untreated mice received an i.p. injection of saline solution.

Behavioral measures

Behavioral characterization was performed during the light cycle, in a dedicated sound-proof room. Testing equipment was thoroughly cleaned between each session. Behaviors were videotaped to be scored by a trained observer blind to treatment conditions (Smart, Panlab, Barcelona, Spain). Two different cohorts of mice were used for the two behavioral tasks.

Well validated tests were used to assess 2 main symptom dimensions of depression: neurovegetative changes (by assessing locomotor activity) and depressive-like behavior (by the sucrose preference test assessing anhedonia) (Vrieze et al. 2013).

Sucrose preference test

Mice were placed in individual cages a few days before the beginning of the experiment.

Briefly, mice were first habituated with two bottles of water for 3 days in individual cages. Then one of the bottles was filled with 2% sucrose solution and basal sucrose preference was measured daily by recording the volume of water and sucrose drunk during 5 consecutive days. After this basal period, mice received an injection of saline or LPS (830 μg/kg, i.p.). BH4 (50 mg/kg) or saline was then injected (i.p.) 21h later. Sucrose preference was measured during the 12h, 24h and 48h period following LPS challenge. The bottle position (right vs. left) was switched every day to eliminate potential biases of place preference. The consumption of each fluid was assessed by weighting bottles and sucrose preference (%) was calculated as: sucrose consumption/(sucrose + water consumption) × 100%.

Locomotion

The day of testing, each mouse was transferred to the open-field apparatus (40 × 40 cm) and was allowed to freely explore it. A video tracking system (Smart, Panlab, Barcelona, Spain) recorded the exact track of each mouse as well as total distance travelled (cm), as a measure of spontaneous locomotor activity for 30 min. Mice were then injected with a saline or an AMPH (3 mg / kg, i.p.) solution in order to elicit locomotion by releasing DA from terminals, and locomotion was recorded for 1 more hour (Vezina 2004).

Biochemical measurements

Two other cohorts of mice were used for biochemical analyses. Mice were sacrificed by decapitation after deep anesthesia using isoflurane (5%), brain was quickly extracted and the striatum was rapidly removed, dissected on ice, and stored at -80°C until further use.

Brain amino acids content

Striata were homogenized in 7 volumes of Tris buffer and sonicated. Quantitative analysis of Arg, Phe and Tyr in striatum homogenates was performed using the EZ: fast kit (Phenomenex, Torrance, CA). This procedure involves a solid phase extraction step, derivatization and subsequent liquid/liquid extraction of the derivatized amino acids. Briefly, 100 µL of homogenates were mixed with 100 µL of internal standards and passed through sorbent tips binding amino acids. Following a wash step with 200 µL of N-propanol, amino acids were eluted with 200 µL of NaOH/N-propanol, 3/2, v/v). The samples were then incubated for 1 min with 50 µL of derivating reagent (propyl chloroformate). The products of derivatization were then extracted with 100 µL of Iso-octane by vortexing vigourously for 5 s. The upper organic layer was transferred to a glass vial, completely evaporated to dryness under a stream of nitrogen and re-dissolved in 100 µL of mobile phase to be analyzed on a UHPLC-MS/MS system (Waters corp., Mildford, MA). Analytes were separated on a 2.5 x 100 mm Synergi Hydro RP column, 2.5 µm particle size coupled with a Security Guard Cartridge AQ C18 4 x 2.0 mm (Phenomenex, Torrance, CA). Column temperature was set at 35°C. The mobile phase consisted of 10 mM ammonium formate in water (A) and methanol containing 10 mM ammonium formate (B). The flow rate was set at 0.44 mL/min. The following gradient was used: 68-83 % of B (0-3.25 min), 83-68 % of B (3.25-3.26 min) 68 % of B (3.26-4.25 min). Data were acquired with MassLynx 4.1 software and quantification was determined by TargetLynx software.

Brain BH4 content

BH4 concentrations were determined by high performance liquid chromatography (HPLC) and quantified using electrochemical detection as previously described (Latini et al. 2018). Weighed striata were homogenized in a mix of HCl 2.5 mM, dithioerythritol 6.5 mM and ascorbic acid 50 mM at 4°C, centrifuged (10 min x 16 000 x g at 4°C) and then supernatant was stored at -80°C. Twenty µL were injected in the HPLC system, equipped with a Waters Atlantis dC18, reverse phase column (4.6 × 250 mm; 5 µm particle), and a DECADE II detector (Antec Leyden, The Netherlands) using a glassy carbon electrode set at +500 mV. The mobile phase, consisting of 6.5 mM NaH_2P0_4, 6 mM citric acid, 1 mM sodium octyl sulfate, 2.5 mM diethylenetriaminepentaacetic acid, 160 µM dithiothreitol and 8% acetonitrile, was pumped at 0.7 mL/min. BH4 was quantified against a daily injected standard and expressed as pmols/g of wet tissue.

Brain DA content

Striata were homogenized in 0.5 mL of a buffer containing 12 mM $HClO_4$, 56 µM EDTA, 0.26 mM $Na_2S_2O_5$ and 3 mM octanesulfonic acid with a Potter-Elvehjem tissue grinder at 4°C. After centrifugation (20 min x 30 000 x g at 4°C), the supernatant was stored in 100 µL aliquots at -80°C until use. Contents of DA and its metabolites dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid

(HVA) were measured by HPLC coupled with electrochemical detection, as previously described (Monchaux De Oliveira et al. 2021).

Gene expression analysis by quantitative real-time PCR

The mRNA levels were determined in the striatum for markers of inflammation (IL1β, IL6, TNFα, MCP-1) and BH4 synthesis (GCH1, GFRP, DHFR, SR, PTPS). Total RNA was extracted with Trizol (Sigma) and 2 µg from each sample was reverse-transcribed into cDNA using using oligo dT primers/RNA mix and then inactivated at 65°C for 5 min and chilled on ice. One hundred ng of the resulting cDNA was amplified in duplicate and gene expressions were measured using the Taqman Universal PCR Master Mix (Applied Biosystems) and appropriate FAM-labeled Taqman primers as previously described (Monchaux De Oliveira et al. 2021). The assay IDDs for the target genes were: GCH1 (Mm01322973_m1), SR (Mm00488430_m1), PTPS (Mm00478494_m1), GFRP (Mm00622819_m1), DHFR (Mm00515662_m1), IL1β (Mm00434228_m1), IL6 (Mm00446190_m1), TNFα (Mm00443258_m1), MCP-1 (Mm00441242_m1), and for the house keeping gene GAPDH (Mm99999915_m1). Data were analyzed using the comparative threshold cycle and results were expressed as relative fold change.

Statistics

Data are reported as means + SEM. All parameters were analyzed using Kruskall-Wallis one-way ANOVA between independent groups or by two-way repeated measures ANOVA, with Treatment as a between-subjects factor and Time as a within-subjects repeated-measures factor (SigmaStat 3.5). All *post hoc* comparisons were made using Bonferroni test. The threshold for statistical significance was set at $p<0.05$.

Results

In order to verify the effectiveness of LPS treatment, we measured body weight change 24h after NaCl or LPS injection. As expected, there was a significant weight loss in LPS-treated mice as compared to controls (26.7 ± 2.0 vs. 29.5 ± 4.0 g, *p<0.05*). BH4 treatment did not affect body weight, neither in the controls (28.3 ±1.9g) nor in the LPS treated mice (25.4 ±0.8 g).

1- Behavioral response to LPS and BH4 administration

As a first aim, we studied behavioral outcomes relevant to depressive symptom dimensions reported to be altered by LPS challenge (Dantzer et al. 2008, Frenois et al. 2007). As expected, results showed that mice exhibited a significant reduction in sucrose preference 12h and 24h following LPS injection, as compared to controls (**Fig.2A**) (*p<0.05*), associated to a significant reduced sucrose consumption (data not shown). Moreover, the spontaneous locomotion was significantly reduced by a half in the open-field test in mice challenged by LPS (**Fig.2B**) (*p<0.05*). The AMPH-stimulated locomotion was also measured in both control and LPS-treated mice, 24h after injection. Even if the four experimental groups displayed the same habituation-related significant decrease in locomotion during the 30 min-measure of spontaneous locomotion, they exhibited dramatic differences in response to AMPH. Control mice displayed an expected and rapid increase in locomotion, observed from the 5th min after AMPH challenge and persisting during the 60-minutes recording (AMPH effect:

p<0.05). However, the response to AMPH was totally blunted in LPS-treated mice since no significant change in locomotion was observed (p>0.05), whatever the time considered (**Fig.2C**). Globally, 24h after the challenge, LPS treated mice showed a 69% reduced distance moved during the 60 min following AMPH injection, as compared to control mice (AUC; *p<0.001*).

Regarding the effect of BH4 on these parameters, results showed that, whereas BH4 pre-treatment did not affect the sucrose preference either in control or in LPS challenged mice (**Fig.2A**), it restored the AMPH-induced locomotor dysfunction in the LPS group (**Fig.2C**), with a significant effect of AMPH observed from the 10^{th} min. On the other hand, acute BH4 treatment did not affect locomotor activity 3h after injection, with a spontaneous (**Fig.2B**) and AMPH-stimulated locomotion (**Fig.2C**) similar to control mice.

In summary, LPS induced a delayed anhedonia and hypolocomotion under AMPH stimulation. However, BH4 treatment, administered 21h after LPS challenge, was able to restore the locomotor function but had no effect on anhedonia, suggesting that distinct pathways are involved in BH4 mediated effects on behavior.

2- Modulation of the striatal BH4 pathway by LPS and BH4 administration

Because of the results obtained with locomotion measure, we focused our biochemical and neurochemical analyses on the striatum, a brain area involved in the regulation of motor function (Dunnett 2005).

LPS challenge induced a significant increase in the mRNA expression of GCH1, showing activation of the *de novo* synthesis pathway of BH4 (**Table 1**). However, expression of the two downstream synthesis enzymes, i.e., PTPS and SR, and of both regulatory proteins GFRP and DHFR, was not changed in LPS-treated mice as compared to control mice. This LPS-induced increase in GCH1 expression was not modified by the BH4 treatment, whereas BH4 drastically reduced the expression of GFRP in control mice 3h after its administration (*p<0.05*).

Striatum levels of BH4 and plasma levels of BH4 and BH2 were unchanged in LPS-challenged animals as compared to controls (plasma BH2 (nM): 161.4 ± 15.5 vs. 239.4 ± 29.5; plasma BH4 (nM): 106.5 ± 32.9 *vs.* 64.8 ± 10.8). Administration of an acute dose of BH4 significantly increased levels of BH4 in the striatum of control and LPS mice (*p<0.05*) (**Fig.3**).

As depicted in **Table 2**, the main changes induced by LPS challenge concerned the levels of L-aromatic amino acids in striatum, with increased levels of Phe (17%; *p<0.05*) and an increased Phe/Tyr ratio (30%; p<0.05), whereas Arg and Tyr levels remained unchanged.

To resume, despite activation of the *de novo* synthesis pathway, LPS challenge did not induce detectable changes in BH4 content in the striatum, 24h after injection, but resulted in an increased Phe/Tyr ratio, suggesting reduced BH4 activity. BH4 treatment was effective to increase striatal BH4 levels, but had no effect on the BH4 synthesis pathway after LPS challenge, since no change in the gene expression of the GCH1, PTPS or SR was observed.

3- Modulation of striatal DA levels by LPS and BH4 administration

As BH4 is the obligatory cofactor for DA synthesis, we next investigated whether the reduced BH4 activity, illustrated by an increase in the Phe/Tyr ratio, could compromise production of DA. Results showed reduced levels of DA in the striatum of mice 24h after LPS challenge (-56 %; *p<0.05*) (**Fig.4A**). However, this decrease was fully restored by the BH4 pre-treatment in the LPS treated mice, whereas BH4 injection had no effect on DA levels in control mice, 3h after injection. Changes in DOPAC levels (**Fig.4B**) globally followed the pattern observed for DA levels, but without reaching statistical significance, probably due to a higher spread of data. On the other hand, HVA levels were identical in all experimental groups (**Fig.4C**).

4- Modulation of brain pro-inflammatory cytokines by LPS and BH4 administration

As expected, LPS challenge induced a significant rise in the striatum expression of IL-1β (**Fig.5A**) and TNF-α 24h after injection (**Fig.5B**; *p<0.05*), compared to control mice, whereas IL-6 expression was identical to basal values (**Fig.5C**). MCP-1 expression was also greatly increased 24h after LPS injection (**Fig.5D**; *p<0.05*). BH4 pre-treatment was found to modulate the cytokine response to LPS challenge. Indeed, whereas BH4 had no effect on mRNA expression of the four cytokines measured in control mice, 3h after treatment, it significantly dampened the expression of IL-1β and TNF-α, and to a lesser and non-significant extent MCP-1 in LPS treated mice (*p<0.05*).

Discussion

The aim of this study was to characterize the involvement of the BH4 pathway in an immune challenge induced by LPS in mice, and to describe the impact of an acute BH4 supply on the behavioral and neurochemical associated changes. Behavioral alterations and neurobiological corelates were assessed 24h after LPS challenge, when sickness behavior is minimal, while depressive-like behavior persists (Frenois et al. 2007, O'Connor et al. 2009). BH4 was administered 21h after LPS challenge, i.e., at the time when the reduction of its bioavailability, which results from the initial induction of inflammatory cytokines by LPS, is believed to contribute to LPS-driven behavioral alterations. Moreover, we previously showed that brain levels of BH4 significantly increased 3h after an acute peripheral administration of BH4 at a dose of 50 mg/kg (Fanet et al. 2020). As previously described, LPS challenge led to induction of GCH1 expression 24h after injection, meaning activation of the *de novo* synthesis of BH4 (Mori et al. 1997, Werner et al. 1993, 2011). However, mice subjected to LPS exhibited a higher Phe/Tyr ratio as compared to controls, suggesting a reduced activity of the BH4 pathway, without changes in brain BH4 levels. LPS challenge also induced a drastic reduction of DA levels in the striatum, that was fully restored by the BH4 treatment. LPS induced the expected delayed anhedonia (Frénois et al. 2007, O'Connor et al. 2009) and blunted the hyperlocomotor response to an AMPH injection. The main result presented here is that BH4 treatment, administered during the phase of inflammation resolution, restored the AMPH-induced hyperlocomotion, suggesting an impact on striatal dopaminergic neurotransmission. By contrast, the BH4 treatment had no effect on anhedonic-like behavior. In parallel, our results show that the BH4 treatment reduced the pro-inflammatory cytokines expression induced by LPS.

BH4 is essential for numerous physiological processes such as neurotransmission, vascularization, metabolism, inflammation, glucose homeostasis and oxidative status (Werner et al.

1998, 2002, 2011). The regulation of the GCH1 activity is the first-line mechanism in the maintenance of BH4 homeostasis (Nagatsu et al. 1989). GCH1 is reported to be colocalized with the monoamine-containing cells, with variable enzyme activity according to brain structure, the highest being found in rat striatum and hypothalamus (Dassesse et al. 1997, Lentz and Kapatos 1996, Nagatsu et al. 1995). Increasing evidence has shown that GCH1 and one of its regulatory protein, the GFRP, are up-regulated under inflammatory condition (Werner et al. 1993, 2011). We observed a significant induction of GCH1 mRNA, 24h after LPS challenge, without change in PTPS, SR or DHFR, as previously described (Kaneko et al. 2003). This shows that the *de novo* pathway leading to BH4 synthesis was recruited after LPS challenge, without implementation of the salvage pathway. However, this GCH1 induction was not associated with concomitant increased BH4 levels in the striatum, 24h after LPS challenge. Our results also showed a significant decrease in brain GFRP mRNA expression 3h after the acute BH4 administration. GFRP mediates BH4 feedback inhibition and Phe feedforward stimulation of GCH1 activity *via* direct interactions with the enzyme (Yoneyama and Hatakeyama 1998). It has been previously described that BH4 administration at our dose induced a reduction in GFRP mRNA expression in the brain of hyperphenylalaninemic ENU1/2 mice (Scherer et al. 2018). In addition, converging studies based on *in vitro* manipulations of GFRP expression globally showed that BH4 levels inversely correlate with GFRP mRNA levels, indicating a fine tuning in BH4 production from GFRP (Kalivendi et al. 2005; Werner et al. 2002). However, the mechanism leading to the reduction in genic expression of GFRP remains not fully understood. We can speculate that such a reduction in GFRP mRNA expression results in reduced GFRP protein content and lower formation of GCH1-GFRP complex. Then, by reducing GFRP expression, accumulation of BH4 is potentially maximized due to down-regulation of the feedback inhibitory mechanism. However, further studies are required to support these hypotheses. In that context, it would be particularly useful to follow the time course of GCH1 activation and BH4 production over the 24h post-LPS injection, in order to better characterize the response of this pathway to an inflammatory stimulation.

Production and use of BH4 in inflammatory condition are complex and highly regulated processes (Fanet et al. 2021, Kim and Park 2010). As a cofactor with great affinity for NOS, BH4 is preferentially used after LPS challenge to produce NO and fight the infection (Teigen et al. 2007). Evidence indicates that cytokines promote oxidative stress and trigger the high output of reactive oxygen species (ROS) throughout induction of inducible NOS for NO production (Bogdan 2015, Coleman2001). This increase in oxidative stress can then contribute to oxidative reduction of BH4, highly redox sensitive, into BH2, leaving less BH4 available for DA synthesis. In line with this, the increased Phe levels and Phe/Tyr ratio observed in striatum of LPS treated mice suggest a less availability of BH4 that could in turn impair the functioning of L-aromatic acid enzymes such as PAH and TH. Consistent with this, we showed that the DA levels in striatum of mice exposed to LPS challenge were reduced 24h after injection. It has been shown previously that peripheral LPS challenge can elicit brain DA transporter activity, leading to increased DA catabolism (Van Heesch et al. 2014). Our results showed that the BH4 treatment restored DA tissue levels in LPS-treated mice, with no effect on DA turnover. This was associated with a significant increase in brain BH4 levels. It was previously reported that BH4 enhances the release of DA in rat brain when administered locally by retrodialysis (Koshimura et al. 1990, Mataga et al. 1991). A dose-dependent increase in striatal extracellular DA concentrations was also described after peripheral BH4 supply (Tsukuda et al. (1994). Although the exact mechanism is unknown, data using TH inhibitors showed that BH4 directly stimulates DA release, independently of its cofactor role (Koshimura et al. 1994). Our data suggest that BH4 administration is able to

counteract the LPS-induced decrease in cerebral DA levels, probably through a direct action on DA release (Fanet et al. 2020).

We wondered if there was an interplay between the reduction in DA striatal levels induced by LPS and changes in locomotor activity, as assessed using the open-field test in spontaneous and AMPH-induced conditions. Results showed that, 24h after LPS challenge, a reduced locomotor activity persisted, in accordance with previous work (Biesmans et al. 2013). In order to enhance the striatal dopaminergic transmission, mice were injected with AMPH, which is known to induce a rapid and robust locomotor hyperactivity (Vezina 2004 for review). Our results showed that, although a significant hyperlocomotion was observed in control mice, the locomotor activity under AMPH challenge was totally blunted in LPS mice, suggesting a DA-related impairment. We also showed that, although BH4 had no effect on the spontaneous activity of control and LPS mice, the cofactor completely restored the AMPH-induced locomotion to control levels. An increased hyper-locomotion induced by methamphetamine challenge after peripheral BH4 analog injection in mice (100 mg/kg) has been previously described (Asami and Kuribara 1989). The authors postulated that BH4 enhances the methamphetamine effect through its action as a cofactor for TH, eliciting DA synthesis. Altogether, these data suggest that LPS challenge leads to a reduction of DA available for release under high demand because of a reduced capacity of synthesis due to lower BH4 availability. The supply of BH4, by increasing its level within the brain, could then restore the optimal activity of DA synthesis and release in response to the AMPH stimulus (Fanet et al. 2020). It has been reported that supplementation with BH4 can stimulate and stabilize TH activity (Nagatsu et al. 1994, Ota et al. 2007, Thöny et al. 2008). Other data showed that administration of BH4 is able to restore the DA content without affecting TH activity in the striatum of Parkinson model of MPTP-treated mice (Kurosaki et al. 2019). However, complementary studies should be done to measure TH activity in our experimental conditions.

Moreover, it is well described that inflammatory conditions are associated with the extracellular release of ATP, which functions as an autocrine and paracrine signaling molecule (Dosch et al. 2018 for review). This release of ATP could lead to a loss of function of ATP-dependent specific mechanisms such as vesicular uptake and storage of neurotransmitters *via* VMAT2 (Mulvihill 2019). The function of VMAT2 is then intrinsically linked to the vacuolar H^+-ATPase that ensures electrochemical gradient across the vesicular membrane and loading of neurotransmitters into vesicles. Thus we can hypothesize that the reduced DA levels observed after LPS challenge can be associated to a lack of function of v-ATPase resulting from a deficit in ATP. By contrast, it has been shown that sepiapterin supplementation, a precursor of BH4, increased ATP production in activated Gch1-ablated T cells (Cronin et al. 2018, 2019). We could then propose that BH4, by restoring ATP levels, could help to package and protect DA into secretory vesicles in an ATP-dependent mechanism and to restore striatal DA levels after an inflammatory challenge.

It is interesting to note that our results showed no effect of BH4 treatment on LPS-induced anhedonia. It has been shown that consummatory pleasure that is measured in the sucrose preference test is not associated with the dopaminergic system in the striatum (Wang et al. 2021). More generally, impaired mesolimbic DA does not appear to affect consummatory pleasure or the reinforcement value of food (Salamone et al. 2016). In line with this, our results show that BH4 acts on dopaminergic function by modulating locomotor activity but not anhedonia, in which alterations in glutamate signaling and GABA transmission have been described (Wang et al. 2021). Consistently, we previously showed that acute BH4 treatment in control mice induced an increase in AMPH-stimulated DA release

and in motivated behaviors in which DA function is involved (Fanet et al. 2020). This suggests that, in inflammatory conditions, BH4 can positively impact locomotion, by acting as DA releaser and in turn counteracting the deleterious effect of LPS. BH4 could then be considered as a potential add-on molecule, helping to maintain or restore dopaminergic neurotransmission in neuroinflammatory conditions. It would be very interesting to test the effect of BH4 supply on other neurovegetative symptoms dependent of DA function and classically observed in depression, modeled for example by alterations in nesting and splash test in mice (Dournes et al. 2013, Yalcin et al. 2008).

Results of the measure of cytokines mRNA in the striatum support the idea of a beneficial impact of BH4 supply in inflammatory condition. As previously described in the hippocampus and the hypothalamus, we observed that the LPS challenge induced a huge striatal expression of the pro-inflammatory cytokines, mainly IL1-β and TNF-α that remained significantly increased 24h after (André et al. 2008). A significant elevation of the chemoattractant MCP-1 remained also at that time, indicating that mild neuroinflammation is still present (Biesmans et al. 2013). Interestingly, our results showed for the first time that the acute BH4 treatment administered during this late phase of inflammation greatly attenuated the increased expression of pro-inflammatory cytokines that persisted 24h after LPS challenge. Such a down regulation of pro-inflammatory cytokines has been described after BH4 treatment in a murine model of pancreas transplantation (Oberhuber et al. 2015). This was associated to a gain in the cell viability of the transplant and to a prolonged recipient survival. Other studies showed that a deficit in BH4 was associated to reduced GCH1 activity leading to cardiac allograft rejection (Ionova et al. 2010), whereas exogenous BH4 supply diminished allograft rejection, possibly by lowering expression of the pro-inflammatory IL-2 (Brandacher et al. 2006). Interestingly, results from immunotherapy studies using recombinant human cytokine IL-2 as treatment reported increased occurrence of severe neuropsychiatric symptoms (Dantzer et al. 2008). However, the involved mechanism remains to be elucidated and further studies will be needed to unravel the complex effect of BH4 on inflammation.

Conclusion

This study provides further evidence for the understanding of the involvement of the BH4 pathway in the behavioral and neurochemical response to an immune challenge. Our results showed that 24h after injection, an LPS challenge decreased activity of the BH4 pathway, reduced DA brain levels and impaired the locomotor response to AMPH stimulation. However, the acute treatment with BH4 during the late phase of LPS-induced inflammation, was effective to restore DA levels and to normalize locomotion in inflammatory condition. This might be linked to a BH4-mediated attenuation of the cytokine response to LPS challenge. As a whole, these data suggest that BH4 could be an interesting compound helping to reduce neuroinflammation and to improve associated DA-related behavioral alterations. More studies are needed to address this question. It would be of interest to test the effect of a BH4 in preclinical models exhibiting inflammatory-related depressive-like symptoms. We recently showed, for example, that mice chronically exposed to a high fat diet expressed neurovegetative and depressive-like behaviors, and increased circulating and central levels of inflammatory factors (Cardinal et al. 2021). Moreover, changes in monoamines levels and in BH4 pathway were identified in different brain areas, suggesting that BH4 could be a potential player in restoring these inflammation-driven alterations.

Acknowledgements

This work was supported by the Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Envirronnement (INRAe, France) (Département AlimH), the Marcel Dassault Prize for Research in Mental Disorders (Fondation FondaMental) and the Fondation pour la Recherche Médicale (FRM, ENV202003011543). Analyses of amino acids were performed at the Plateforme Analytique, Institut sur la Nutrition et les Aliments Fonctionnels, Université Laval, Québec (Canada). H. F. was supported by a doctoral fellowship from the INRAe and from the Université de Bordeaux. Authors thank the animal technicians for animal care.

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Table 1. Relative abundance of mRNA encoding BH4-pathway enzymes in striatum of mice in response to LPS and BH4 treatment.

	Controls n=8	3h post-BH4 n=6	24h post-LPS	
			no BH4 n=8	BH4 50 mg / kg n=8
GCH1	1.0 ± 0.1[a]	1.0 ± 0.1[a]	1.5 ± 0.2[b]	1.7 ± 0.1[b]
PTPS	1.0 ± 0.1[a]	1.2 ± 0.1[a]	1.2 ± 0.1[a]	1.3 ± 0.1[a]
SR	1.0 ± 0.1[a]	1.1 ± 0.1[a]	1.2 ± 0.1[a]	1.1 ± 0.1[a]
GFRP	1.0 ± 0.1[a]	0.4 ± 0.0[b]	1.0 ± 0.1[a]	0.9 ± 0.1[a]
DHFR	1.0 ± 0.2[a]	0.9 ± 0.1[a]	1.3 ± 0.2[a]	1.2 ± 0.1[a]

GCH1, guanosine triphosphate cyclohydrolase-1; PTPS: 6-pyruvoyl-tetrahydropterin synthase; SR: sepiapterin reductase; GFRP: GTP cyclohydrolase feedback regulatory protein ; DHFR: dihydrofolate reductase.

Real-time PCR was performed 3h after saline (Controls) or BH4 treatment (3h post-BH4), or 24h after LPS challenge (830 μg/kg), without (no BH4) or with treatment by BH4 3h before sacrifice (BH4 50 mg/kg). Values are percentages of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) abundance and normalized to the control condition (mean ± SEM). (a,b) For a same row, values with different superscripts are significantly different (*p<0.05*; ANOVA).

Table 2. Striatum L-aromaric amino acid levels 24h after LPS-challenge

	Controls n=8	24h post-LPS n=8
Arginine (μM)	20.2 ± 1.0	21.9 ± 1.
Phenylalanine (μM)	7.1 ± 0.5	8.3 ± 0.2*
Tyrosine (μM)	8.1 ± 0.4	8.0 ± 0.5
Phe / Tyr	0.83 ± 0.02	1.09 ± 0.03*

Amino acids were measured 24h after saline or LPS injection. Data are means ± SEM.
*Significantly different from controls (*p<0.05*; ANOVA).

Figure caption

Fig.1

(**A**) **LPS-induced cytokines production induces activation of the three consecutive enzymes GTP cyclohydrolase-1 (GTPCH)**, 6-pyruvoyl-tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR) (*de novo* synthesis pathway (in grey)), leading to BH4 synthesis. GTPCH activity is modulated by the interaction of GTP cyclohydrolase feedback regulatory protein (GFRP) and effectors molecules, BH4 and phenylalanine (Phe). BH4 is rapidly oxidized in BH2 which is subsequently reduced back to BH4 by the enzyme dihydrofolate reductase (DHFR), representing a salvage pathway. BH4 is used as a cofactor for nitric oxide synthases (NOS), phenylalanine hydroxylase (PAH) and tyrosine hydroxylase (TH) activity for the synthesis of nitric oxide (NO), tyrosine (Tyr) and dopamine (DA), respectively, from precursor amino acids (arginine (Arg), phenylalanine (Phe), Tyr).

(B)Timeline of the experiments: Mice received an injection (i.p.) of either NaCl 0.9% (Controls) or LPS (830 μg/kg) at T0. BH4 (50 mg/kg) or NaCl was administered (i.p.) 21h later. The four experimental groups (i.e., NaCl; NaCl+BH4; LPS; LPS+BH4) were then tested 24h after for behavior (locomotion in open-field and sucrose preference test) and were sacrificed for central and peripheral analyses. Different cohorts of mice were used for the behavioral measures and for biochemical analyses.

Fig.2 Effect of BH4 treatment on sucrose preference test and locomotion in mice challenged with LPS.

Sucrose preference (**A**) was measured as % of sucrose consumption, 12h (T+12h), 24h (T+24h) and 48h (T+48h) after LPS challenge (830 μg/kg); BH4 (50 mg/kg) or NaCl was then injected (i.p.) 21h following LPS injection. Data are expressed as mean + SEM (n=10 per group). (a,b) Values with different superscripts are significantly different (*p<0.05*; ANOVA for RM).

Spontaneous locomotion (**B**) was measured 3h after saline (Controls) or BH4 treatment (3h post-BH4), or 24h after LPS challenge (830 μg/kg), without (no BH4) or with treatment by BH4 3h before sacrifice (BH4 50 mg/kg). Following this, mice received an injection of amphetamine (AMPH, 3 mg / kg, i.p.) and locomotion was recorded for 60 min (% of spontaneous activity) (**C**). Data are means + SEM (n=10 per group).

(A): a≠b, *p<0.05*; ANOVA-RM.

(B): a≠b, *p<0.05*; ANOVA.

(C): for each time, values with different superscripts (a,b) are significantly different (*p<0.05*, ANOVA-RM).*Significantly different from T0 (*p<0.05*; ANOVA-RM).

Fig.3: Effect of BH4 treatment on BH4 levels in the striatum of mice challenged with LPS.

Striatum levels of BH4 were measured 3h after saline (Controls) or BH4 treatment (3h post-BH4), or 24h after LPS challenge (830 μg/kg), without (no BH4) or with treatment by BH4 3h before sacrifice (BH4 50 mg/kg). Data are means + SEM (n=8 per group). (a,b) Values with different superscripts are significantly different (*p<0.05*; ANOVA).

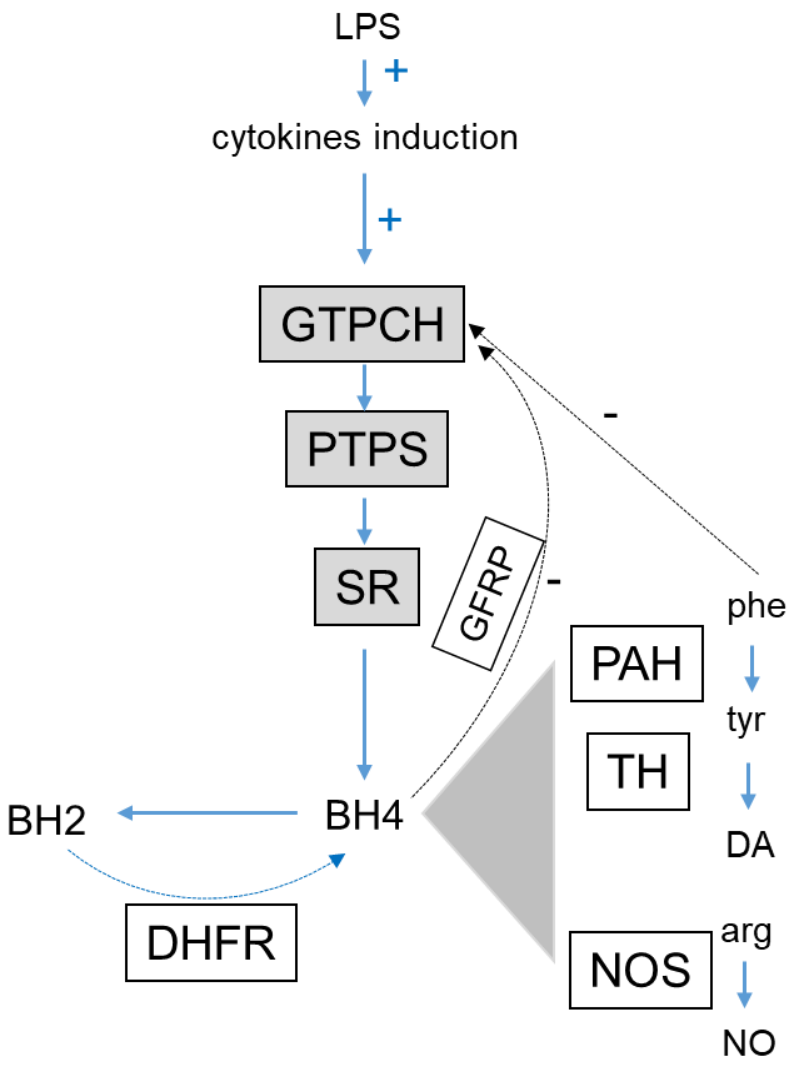
Fig.4: Effect of BH4 treatment on brain DA and its metabolites in the striatum of mice challenged with LPS.

DA (**A**) and main metabolites (DOPAC, **B**; HVA, **C**) were measured 3h after saline (Controls) or BH4 treatment (3h post-BH4), or 24h after LPS challenge (830 μg/kg), without (no BH4) or with treatment by BH4 3h before sacrifice (BH4 50 mg/kg). Data are means + SEM (n = 8 for each group). (a,b) Values with different superscripts are significantly different (*p<0.05*; ANOVA).

Fig.5: Effect of BH4 treatment on pro-inflammatory cytokines mRNA expression in the striatum of mice challenged with LPS.

Real-time PCR was performed 3h after saline (Controls) or BH4 treatment (3h post-BH4), or 24h after LPS challenge (830 μg/kg), without (no BH4) or with treatment by BH4 3h before sacrifice (BH4 50 mg/kg), for interleukin-1beta (IL1β; **A**), tumor necrosis alpha (TNFα; **B**), interleukin-6 (IL6; **C**) and monocyte chemoattractant protein 1 (MCP-1; **D**) measure of mRNA expression. Values are percentages of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) abundance and normalized to the control condition (mean + SEM; n=6 to 8 per group). (a-c) Values with different superscripts are significantly different (*p<0.05*; ANOVA).

A) GCH1 pathway in inflammation



B) Timeline of the experiments

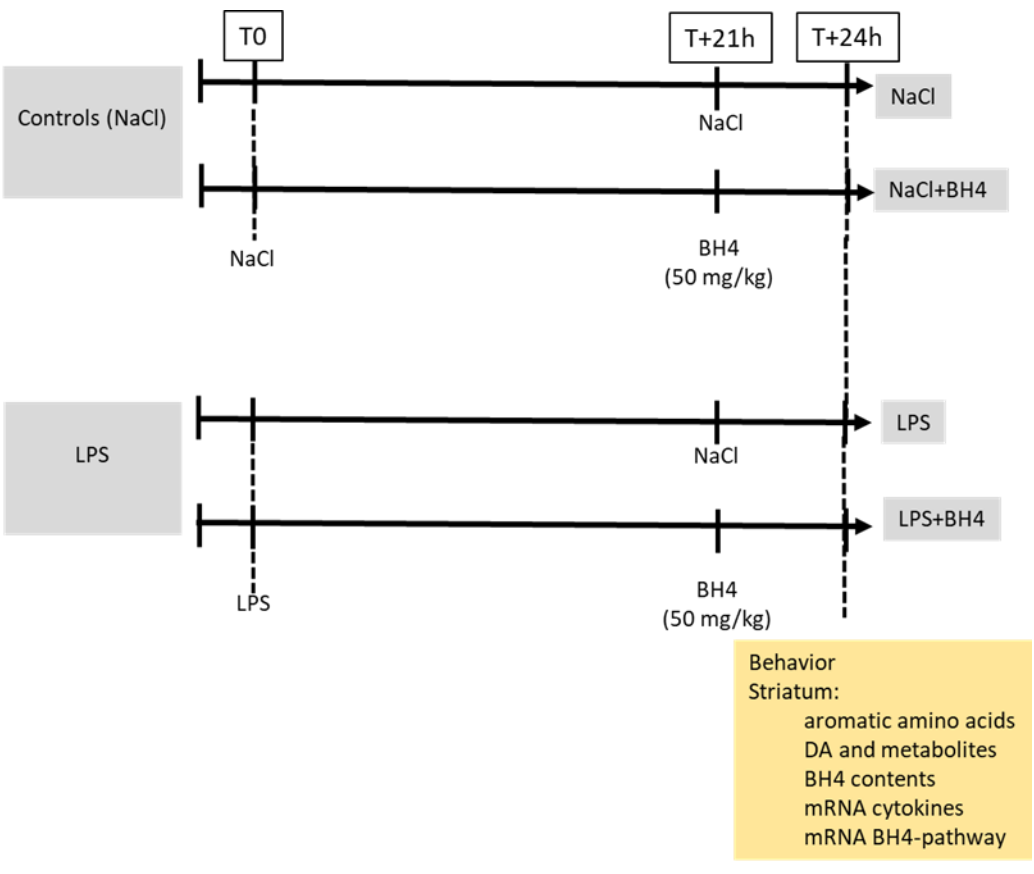
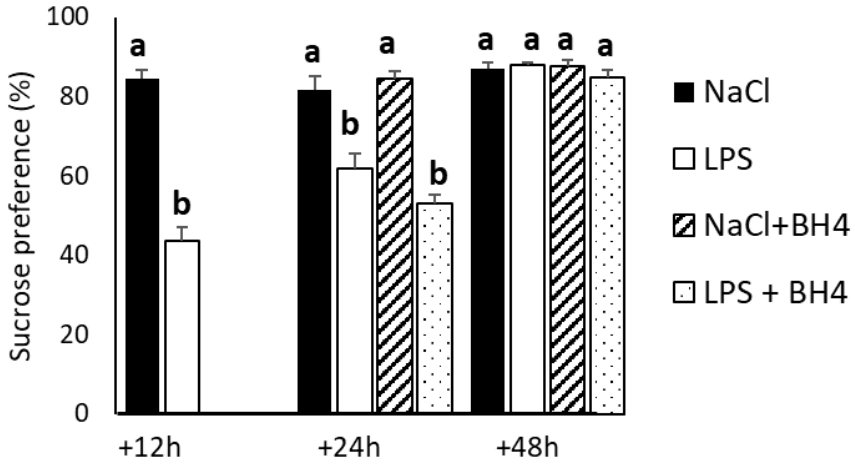
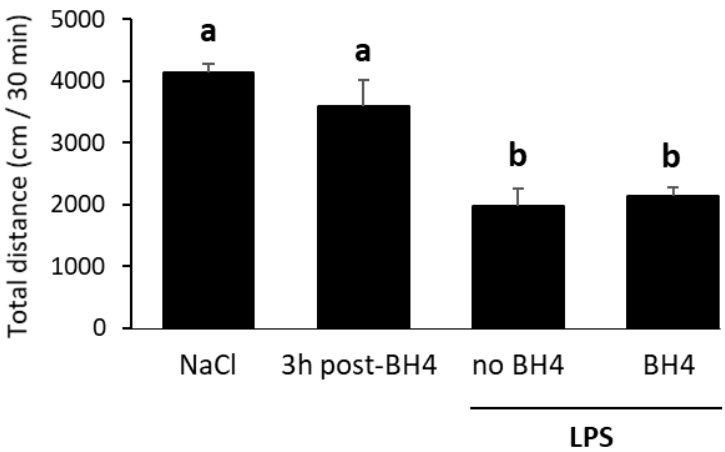


Figure 1

A) Sucrose preference test



B) Spontaneous locomotion



C) Locomotion following amphetamine challenge

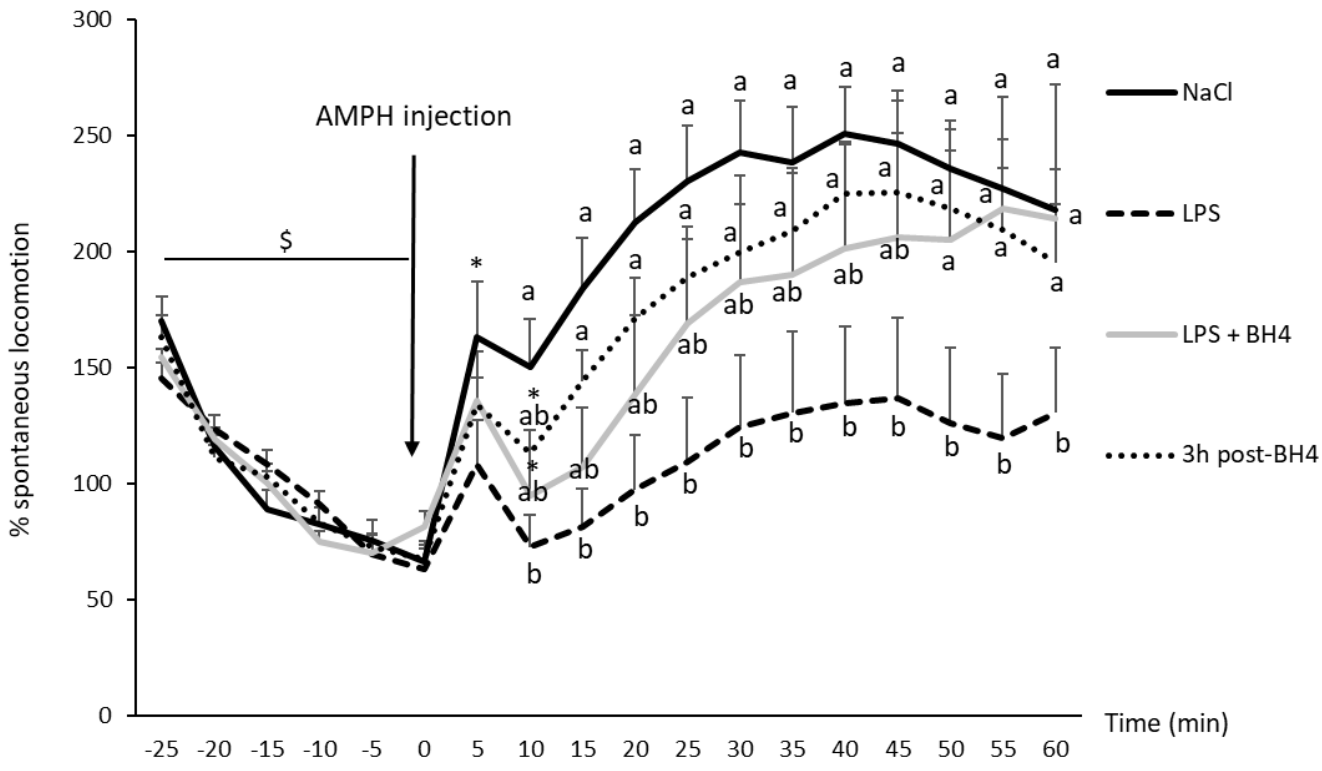


Figure 2

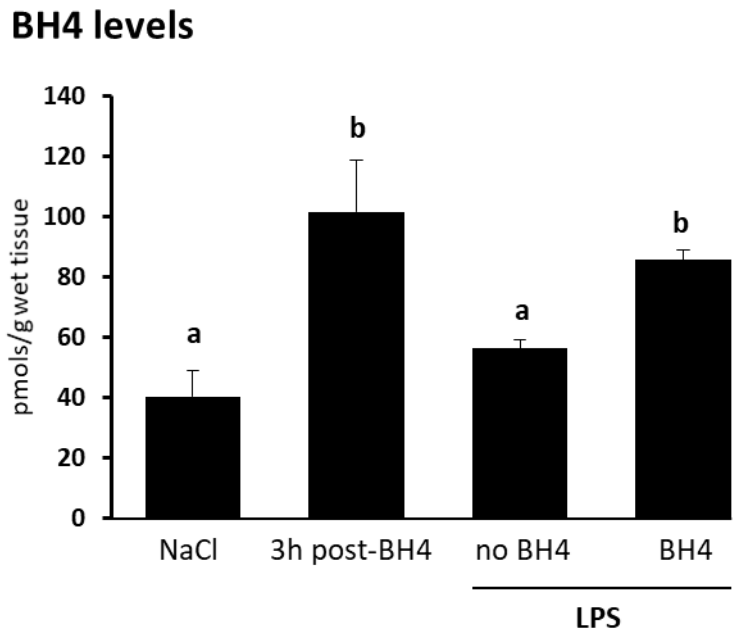


Figure 3

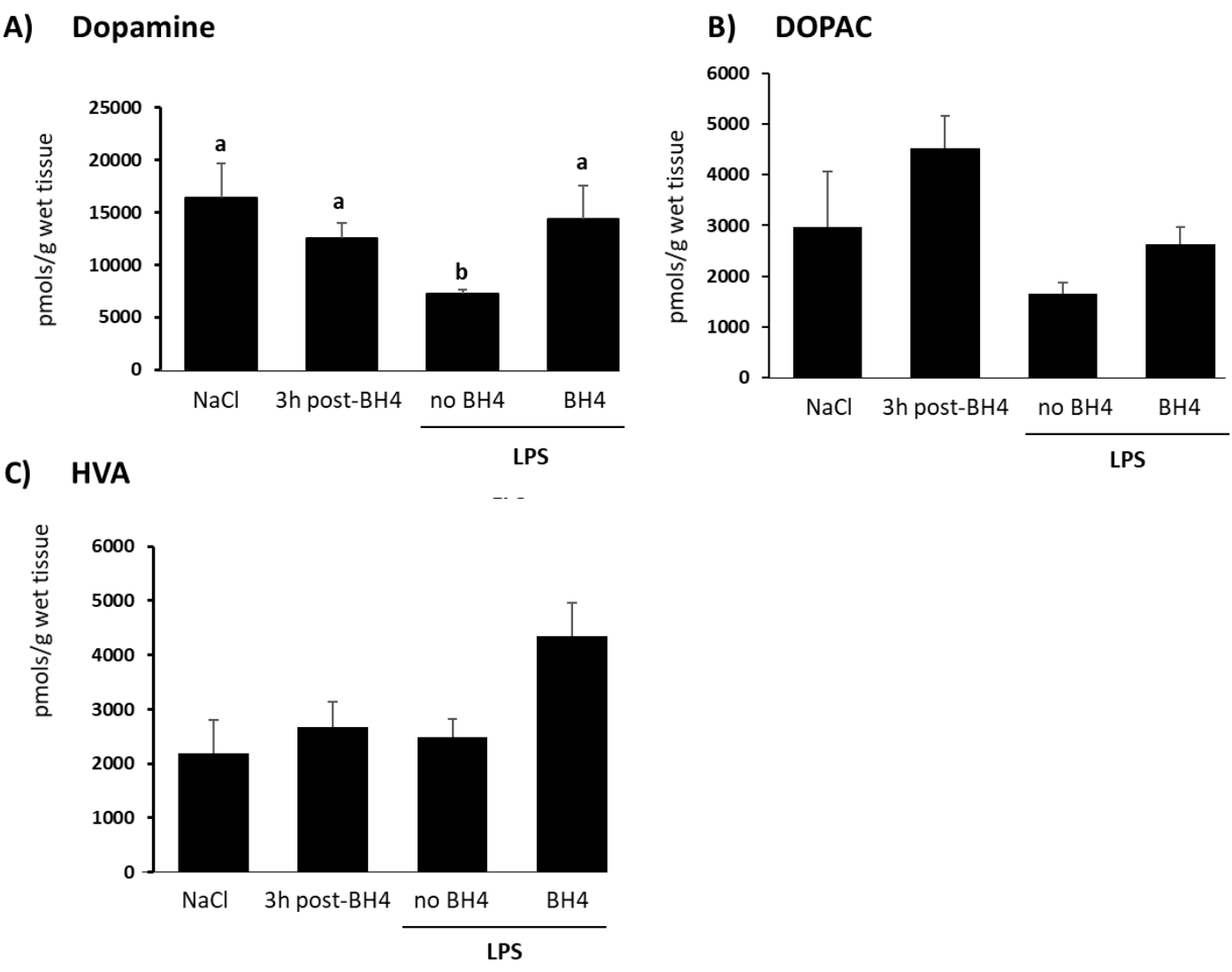


Figure 4

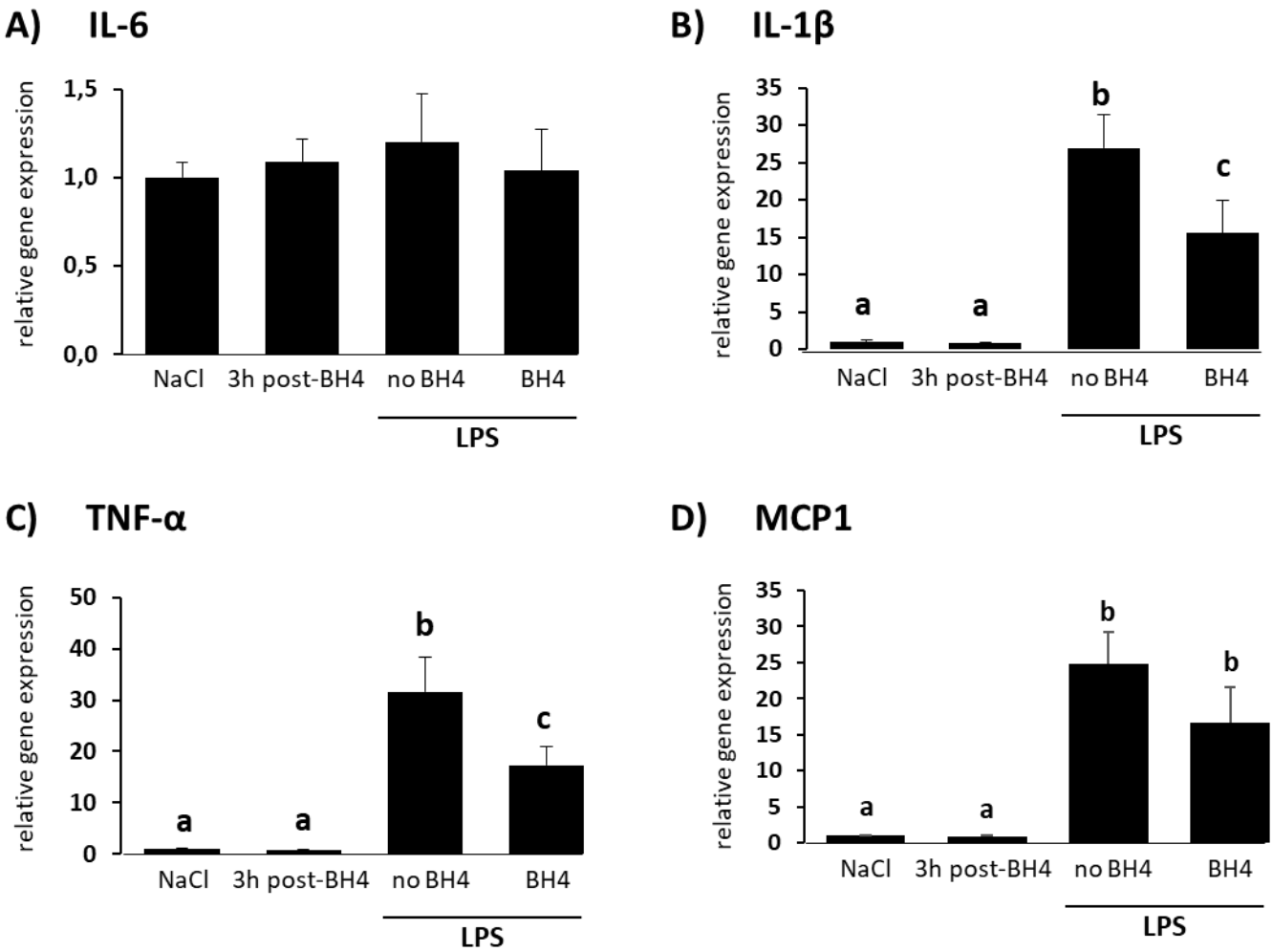

A) IL-6
relative gene expression
1,5
1,0
0,5
0,0
NaCl
3h post-BH4
no BH4
BH4
LPS
B) IL-1β
relative gene expression
35
30
25
20
15
10
5
0
a
a
b
c
NaCl
3h post-BH4
no BH4
BH4
LPS
C) TNF-α
relative gene expression
50
40
30
20
10
0
a
a
b
c
NaCl
3h post-BH4
no BH4
BH4
LPS
D) MCP1
relative gene expression
35
30
25
20
15
10
5
0
a
a
b
b
NaCl
3h post-BH4
no BH4
BH4
LPS

Figure 5