Molecular Genetics and Metabolism xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

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

Tetrahydrobiopterin improves hippocampal nitric oxide-linked long-term memory

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ARTICLE INFO

Keywords:
Memory
LTP
Tetrahydrobiopterin
BH4 salvage pathway

ABSTRACT

Tetrahydrobiopterin (BH4) is synthesized by the combined action of three metabolic pathways, namely *de novo* synthesis, recycling, and salvage pathways. The best-known function of BH4 is its mandatory action as a natural cofactor of the aromatic amino acid hydroxylases and nitric oxide synthases. Thus, BH4 is essential for the synthesis of nitric oxide, a retrograde neurotransmitter involved in learning and memory. We investigated the effect of BH4 (4–4000 pmol) intracerebroventricular administration on aversive memory, and on BH4 metabolism in the hippocampus of rodents. Memory-related behaviors were assessed in Swiss and C57BL/6 J mice, and in Wistar rats. It was consistently observed across all rodent species that BH4 facilitates aversive memory acquisition and consolidation by increasing the latency to step-down in the inhibitory avoidance task. This effect was associated with a reduced threshold to generate hippocampal long-term potentiation process. In addition, two inhibitors of memory formation (N(ω)-nitro-L-arginine methyl ester - L-Name – and dizocilpine - MK-801 -) blocked the enhanced effect of BH4 on memory, while the amnesic effect was not rescue by the co-administration of BH4 or a cGMP analog (8-Br-cGMP). The data strongly suggest that BH4 enhances aversive memory by activating the glutamatergic neurotransmission and the retrograde activity of NO. It was also demonstrated that BH2 can be converted into BH4 by activating the BH4 salvage pathway under physiological conditions in the hippocampus. This is the first evidence showing that BH4 enhances aversive memory and that the BH4 salvage pathway is active in the hippocampus.

1. Introduction

Tetrahydrobiopterin (BH4), a natural pteridine, is an obligatory cofactor for phenylalanine, tyrosine, tryptophan hydroxylases, alkylglycerol monooxygenase, and for all isoforms of nitric oxide synthase (NOS) [1, 2].

Physiological intracellular levels of BH4 are regulated by the combined action of three metabolic pathways, namely *de novo* synthesis, recycling, and salvage pathways. The *de novo via* generates BH4 from GTP through a three-step enzymatic metabolic route starting with the rate-limiting enzyme guanosine triphosphate cyclohydrolase I (GTPCH; GCH1 gene), followed by 6-pyruvoyl tetrahydropterin synthase (PTPS) and sepiapterin reductase (SPR) [3]. Alternatively to the *de novo* synthesis pathway, intracellular BH4 levels can be generated *via* the salvage pathway using sepiapterin and 7,8-dihydrobiopterin (BH2) as metabolic intermediates. Although the salvage pathway is not fully understood, SPR and dihydrofolate reductase (DHFR) appears to be key generating BH4 enzymes [2, 4]. The BH4 recycling pathway is a mechanism that economizes intracellular energy and sustains the appropriate levels of BH4 in tissues with high requirement of this pteridine.

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https://doi.org/10.1016/j.ymgme.2018.06.003
Received 13 March 2018; Received in revised form 9 June 2018; Accepted 9 June 2018

After BH4 participates as a mandatory enzyme cofactor, quinonoid dihydrobiopterin (qBH2) is formed and reduced back to BH4 in a NADH-dependent reaction [3].

As the rate-limiting enzyme for BH4 synthesis, GTPCH regulation takes place at transcriptional levels. Inflammatory mediators, including interferon gamma (IFN-γ), tumor necrosis factor–α (TNF-α), interleukin 1-β (IL-1β), and lipopolysaccharide (LPS) or reactive oxygen species (ROS) induce GTPCH gene expression and activity (for a review see [5]). Under these conditions GTPCH expression/activity can be stimulated up to 100-fold, while PTPS and SPR remain slightly increased. Thus, PTPS becomes the rate-limiting enzyme of the BH4 pathway during inflammation. Consequently, the pseudo metabolic blockage will favor the accumulation of neopterin, an established sensitive biomarker for immune system activation [5–7].

The biology of the pterinergic metabolism in the central nervous system is not fully understood. However, our group has recently unveiled additional properties, apart from those described decades ago. For example, excessive BH4 levels produced by damaged sensory neurons, nerve tissues and infiltrating macrophages, increases pain sensitivity in inflammatory and neuropathic pain models, and the use of SPR inhibitors reduces chronic pain with no detectable side effects, such as vasodilation or neurotransmission failures [8]. The use of such inhibitors, also produced increased levels of sepiapterin in tissues and biological fluids, allowing us to propose this pteridine as a biomarker for the BH4 pathway engagement [8, 9]. Furthermore, we have demonstrated that neopterin is formed and released by rodent and human nerve cells under cellular stress [5, 10]. In addition, we have also demonstrated that that after a single neopterin intracerebroventricular administration, resistance to oxidative damage by activating the antioxidant Nrf2/ARE-linked pathway is promoted [5, 11], inflammation is reduced [5], the activation of inflammasome is inhibited [10], and cognition is enhanced in rodents [12]. Since neopterin is a byproduct of the *de novo* pathway, and increased levels of neopterin will occur together with increased BH4 levels, we hypothesize that BH4 induces similar positive effects in the brain. In addition, after the first seminal paper of Woolf [13], it is understood that pain sensitization shares cellular mechanisms with learning and memory, including the generation of long-term potentiation (LTP) [14]. Therefore we here investigated whether BH4 facilitates cognition and the potencial mechanisms involved in this effect.

2. Experimental procedures

2.1. Mice

Adult male Swiss mice from the Central Animal House of the Center for Biological Sciences, Universidade Federal de Santa Catarina (Florianópolis, Brazil) and adult C57BL/6 J male mice obtained from a reproduction colony from the same institution were used in the present experiments.

2.2. Rats

In addition, adult male Wistar rats from the Central Animal House of the Pharmacology Department of School of Chemical Sciences, Universidad Nacional de Córdoba (Córdoba, Argentina), were also used for behavior experiments and LTP measurement.

All the animals were kept under standard laboratory conditions (12 h light-dark cycle, lights on at 07:00 h, temperature 22 ± 1 °C) with free access to food and water. The experimental protocols were approved by the Ethics Committee for Animal Research (PP00425/CEUA) from Universidade Federal de Santa Catarina, and from the Universidad Nacional de Córdoba (RES-48-2015). All procedures were carried out in accordance with the ARRIVE guidelines, in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All efforts were made to minimize the number of animals used and their suffering.

2.3. Stereotaxic surgery

Adult male mice and rats were anesthetized with ketamine (55–80 mg/kg, intraperitoneally (i.p.), Virbac, Carros, France) and xylazine (11 mg/kg, i.p., Bayer, Leverkusen, Germany) and mounted in a stereotaxic apparatus (Insight® Equipment, São Paulo, Brazil). Stainless steel cannula was implanted unilaterally into the lateral ventricle using the following coordinates relative to bregma: C57BL/6 J mice: AP -0.34 mm, LL ± 1 mm, DV -2.3 mm; Swiss mice: AP -0.5 mm, LL +1 mm, DV -2.5 mm; Wistar rats: AP -0.8 mm, LL +1.5 mm, DV -4.0 mm) [15, 16]. Cannulas were fixed to the skull surface with dental acrylic cement.

2.4. Compound preparation and administration

All compounds were prepared freshly on the day of each experiment and injected intracerebroventricularly (i.c.v.; infusion time 1 min) in conscious animals using a 30-gauge needle by gently restraining the animal. BH4 (4–4000 pmol; 1 μL; Sigma, St. Louis, MO, USA) was diluted in artificial cerebrospinal fluid (aCSF) and protected from light. 8-bromo-cGMP (protein Kinase G activator; 20 nmol; 2 μL; Sigma-Aldrich RBI, Natick, MA, USA); N(ω)-nitro-L-arginine methyl ester (L-NAME; NOS inhibitor; 10 mg/kg; i.p.; Sigma, St. Louis, MO, USA), and MK-801 (non-competitive antagonist of the NMDA receptor; 0.1 mg/kg; i.p.; Sigma, St. Louis, MO, USA) were diluted in saline.

2.5. Open field

The open field test was used to evaluate spontaneous locomotor activity. The animals were placed in the center of the open field arena (40 × 40 cm) and recorded for 10 min in order to evaluate the total distance traveled. The tests were videorecorded and analyzed by the ANY-maze Platform™ as we previously described elsewhere [17, 18].

2.6. Step-down avoidance task

The step-down avoidance task was conducted in an acrylic box (mice: 30 × 20 × 20 cm; rats: 50 × 25 × 25 cm) with a grid floor (bars: 1 mm diameter, spaced 1 cm apart) and an elevated platform (mice: 10 cm^2 surface and 2 cm in height at the center of the floor; rats: 7 × 25 cm surface and 2.5 cm in height at the left side of the floor) as previously described [12]. The animals were placed on the platform and their latency to step-down with the four paws on the grid was recorded. During the training sessions, immediately after stepping-down on the grid, animals received a 0.5 mA, 1.0-s scrambled foot shock. No foot shock was delivered in the testing sessions and the step-down latency (maximum 180 s) was used to measure memory retention. Test sessions were performed 24 h after a single training session to evaluate long-term memory (LTM).

L-NAME (N(ω)-nitro-L-arginine methyl ester; 10 mg/kg; i.p.), MK-801 (dizocilpine; 0.1 mg/kg; i.p.) and 8-bromo-cGMP (20 nmol; i.c.v.) were administered 30 min prior the test session (measurement of LTM at 24 h).

The intensity of the electric shock (0.5 mA) was selected from a curve performed in a separate group of mice (Fig. 1A). Mice received a single 0.2, 0.3, 0.4, 0.5, or 0.6 mA electric shock during the training session, and the intensity chosen was based on the highest latency observed in the test session without reaching the ceiling of 180 s. Same electric stimulus was used for rats.

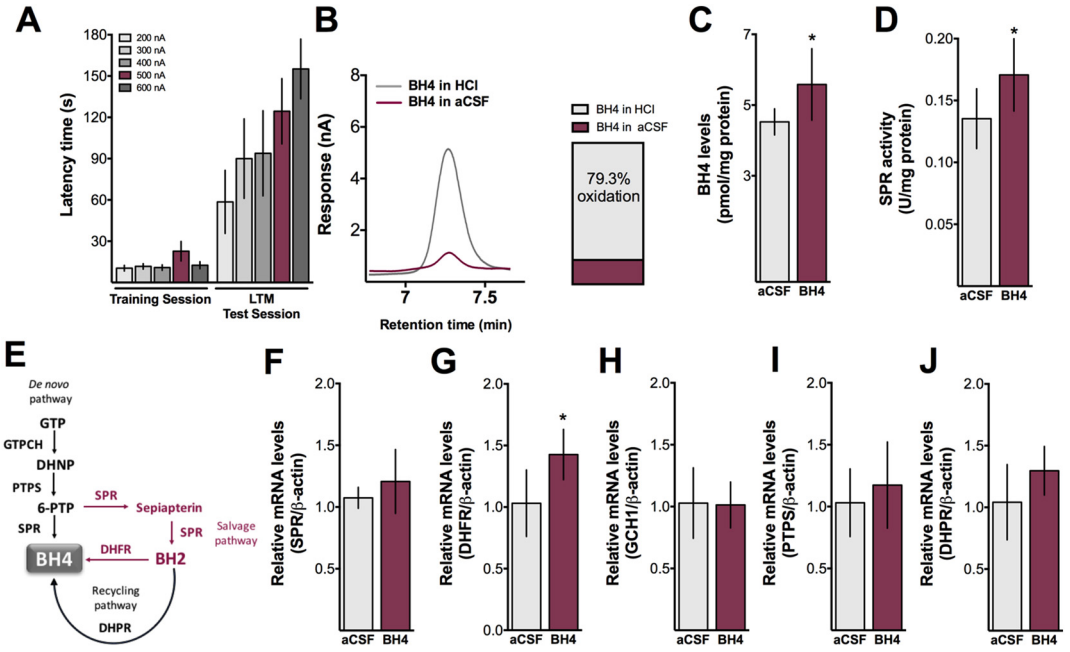

Fig. 1. The tetrahydrobiopterin (BH4) administration increases activity of the hippocampal salvage pathway. Initially, adult Swiss mice received a single 0.2, 0.3, 0.4, 0.5, or 0.6 mA electric shock during the training session of the step-down avoidance task, to select the optimal intensity of the electric shock for further behavioral experiments. The long-term memory (LTM) was assessed 24 h after the training session. The 0.5 mA intensity was chosen since it elicited the higher latency time at LTM without reaching the maximum of 180 s (A). BH4 solution was prepared in 0.1 M HCl (control of oxidation) and artificial CSF (aCSF), and the concentration of BH4 was measured by liquid-chromatography coupled to electrochemical detection before the injections (B). Mice received BH4 (i.c.v.; 4 pmol; 1 μL) and 24 h later the hippocampus was dissected and used to analyze BH4 levels (C), SPR activity measured spectrophotometrically (D) and the gene expression of BH4 biosynthetic enzymes sepiapterin reductase (SPR; F), dihydrofolate reductase (DHFR; G), GTP cyclohydrolase I (GTPCH; H), 6-pyruvoyl tetrahydropterin synthase (PTPS; I) and dihydropteridin reductase (DHPR; J) by qPCR. The BH4 biosynthetic pathways are shown in the scheme (E). Data from the inhibitory avoidance task are presented as median and interquartile ranges. *P $<$ 0.05 (Kruskal-Wallis test). All other values are presented as mean $\pm$ SEM. *P $<$ 0.05 vs aCSF (Student *t*-test for unpaired samples).

2.7. Electrophysiology

Animals were sacrificed 24 h after BH4 (4 pmol; i.c.v.) administration and electrophysiological experiments were carried out using the *in vitro* hippocampal slice preparation [12]. Slices were placed in a recording chamber (BSC-BU Harvard Apparatus, Holliston, MA, USA) perfused with standard Krebs buffer saturated with 95% O_2 and 5% CO_2 (perfusion rate: 1.6 mL/min; bathing solution temperature: 28 °C). Field excitatory postsynaptic potentials (fEPSP) were evoked with a stimulating electrode placed in the perforant path and the recording electrode inserted in the dentate granule cell body layer. Only slices showing a stable response were included. The fEPSP in response to 0.2 Hz pulses (0.5 ms, 10 mA each) were sampled each 5 min during a 20–30 min period (baseline). Once no further changes were observed in the fEPSP amplitude, the stimulation protocol was applied to determine the long-term potentiation (LTP) eliciting frequency threshold. The stimulation protocol consisted of a train of pulse (0.5 ms, 10 mA each) of 2 s duration (tetanus), of increasing variable frequency (5–200 Hz) that was delivered to the perforant path, by an A310 Accupulser Pulse Generator (World Precision Instruments Inc., Sarasota, FL, USA). After the tetanus, a new averaged fEPSP was recorded at 0.2 Hz, and when LTP was not observed, a new stimulation at the next higher frequency was applied. LTP was considered having occurred when the fEPSP amplitude had increased by at least 30% from basal fEPSP and persisted for 60 min. Once LTP was achieved, no further tetanus was given. For each animal, a second hippocampal slice was used to corroborate the threshold to generate LTP by applying tetanus at the same frequency in which LTP was previously elicited. No differences were observed in LTP generation between slices.

2.8. Nitrite measurement

Nitrite levels, a stable metabolite of nitric oxide (NO), were determined using the Griess reaction as previously described by our group [19]. Swiss mice were treated with BH4 (4 pmol; i.c.v.) and/or L-NAME (see Fig. 3A) and euthanized 24 h later, in order to isolate the hippocampi. Brain tissue was homogenized in 20 mM sodium phosphate buffer with 140 mM KCl (pH 7.4) and centrifuged (1000 x *g*; 10 min; 4 °C). The supernatant was and incubated with one volume of the Griess reagent [0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride, 1% sufanilamide, and 2.5% phosphoric acid (all from Sigma, St. Louis, MO, USA)] during ten min at room temperature. Afterwards, the absorbance was read at 550 nm. Nitrite concentrations were calculated from a standard curve of nitrite (Sigma, St. Louis, MO, USA), and expressed as μmol/mg protein.

2.9. L-[^{3}H]-glutamate uptake

L-[^{3}H]-Glutamate uptake was evaluated as previously described [20]. Swiss albino mice were treated with BH4 (4 and 400 pmol; i.c.v.) and euthanized 24 h later, in order to isolate the hippocampi. Slices obtained from hippocampi (400 μm) were washed for 15 min at 37 °C in Hank's balanced salt solution (HBSS) containing, 1.29 mM CaCl2, 136.9 mM NaCl, 5.36 mM KCl, 0.65 mM MgSO4, 0.27 mM Na2HPO4, 1.1 mM KH2PO4, and 5 mM HEPES. Uptake was assessed by adding 0.33 mCi/mL L L-[^{3}H]-glutamate with 100 mM unlabeled glutamate in a final volume of 300 μL. Incubation was stopped immediately after 7 min by discarding the incubation medium. Slices were then submitted to two ice-cold washes with 1 mL HBSS. Slices were solubilized by adding a solution containing 0.1% NaOH and 0.01% SDS and incubated overnight. Aliquots of slice lysates were taken for determination of the

Table 1
Primers used to assess the gene expression.

Encoded protein	Forward sequence	Reverse sequence
β-actin	5′ GCGTCCACCCGCGAGTACAAC 3′	5′ CGACGACGAGCGCAGCGATA 3′
GCH1	5′ TGAGCCCCAGTCCGGGTGAC 3′	5′ GTGCTAACAAGCGCTGCGGC 3′
PTPS	5′ GTCCTTCAGCGCGAGCCACC 3′	5′ CCCGTGTGAGGCCCTGGTGT 3′
SPR	5′ CCGAGTGTGCGGGTGCTGAG 3′	5′ CCAGCGCCCCATCCGACTTC 3′
DHFR	5′AAAGTGGACATGGTCTGGGTA 3′	5′CTGGCTGATTCATGGCTTC 3′
DHPR	5′GCC AGC GTG GTT GTT AAG AT 3′	5′ AAG AGG CCT CCT TCC TTC AG 3′

intracellular content of L-[^{3}H]-glutamate by scintillation counting. Sodium-independent uptake was determined by using choline chloride instead of sodium chloride in the HBSS. Unspecific sodium-independent uptake was subtracted from total uptake to obtain the specific sodium-dependent glutamate uptake. Results were obtained in nmol of L-[^{3}H]-glutamate taken up per mg of protein per minute and expressed as percentage of L-[^{3}H]-glutamate uptake related to control slices.

2.10. Viability assay

MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to evaluate the cellular viability of hippocampal slices obtained from Swiss mice as stated in glutamate uptake experiments (see Fig. 3A). Active dehydrogenases cleavage and reduce the soluble yellow MTT dye into the insoluble purple formazan. Slices were exposed to MTT (0.5 mg/mL; Sigma, St. Louis, MO, USA) and incubated during 3 h. The medium was removed and the slices were diluted in 20% SDS/50% *N,N*-dimethylformamide. The formazan formation was spectrophotometrically followed at 570 nm. Results are indicated as percentage of controls, to which 100% activity was attributed [21].

2.11. BH4 determination

BH4 concentrations were determined by high performance liquid chromatography (HPLC) and quantified using electrochemical detection as previously described with some modifications as follows [8]. Hippocampi obtained from Swiss mice treated with BH4 (4 pmol; i.c.v.; 24 h) were homogenized in 100 µL of 60 mM potassium phosphate buffer and centrifuged (10,000 ×*g*; 10 min; 4 °C). Brain homogenates were precipitated by the addition of one volume (1:1, *v*/v) of HCl 0.1 N containing 6.5 mM dithioerythritol (Sigma; St. Louis, MA, USA). Afterwards, samples were centrifuged (16,000 ×*g*; 10 min; 4 °C) and 20 µL of supernatant were transferred to an HPLC vial for analysis. The HPLC analysis of BH4 was carried out in a HPLC (Alliance e2695, Waters, Milford, USA) by using a Waters Atlantis dC18, reverse phase column (4.6 × 250 mm; 5 µm particle), with a flow rate set at 0.7 mL/min and an isocratic elution of 6.5 mM NaH2P04, 6 mM citric acid, 1 mM sodium octyl sulfate, 2.5 mM diethylenetriaminepentaacetic acid, 160 µM dithiothreitol and 12% acetonitrile, pH 3.0. The temperature of column compartment was set at 35 °C. The identification and quantification of BH4 was performed by an electrochemical detector (module 2465, Waters, Milford, USA) with a voltage of +450 mV. The results were expressed as pmol/mg protein.

2.12. SPR activity

The enzyme activity was measured spectrophotometrically as previously described [22], with some modifications [8]. Hippocampi obtained from Swiss mice treated with BH4 (4 pmol; i.c.v.; 24 h) were homogenized 150 µL of freshly prepared 50 mM Tris-HCL buffer pH 7.5, containing 1.0 mM dithioerythritol and protease inhibitor cocktail (1 tablet/10 mL buffer; cOmplete, ULTRA, Mini, EDTA-free, EASYpack; Roche, USA). After centrifugation (10,000 ×*g* for 5 min at 4 °C) and in order to remove salts and pteridins, sample supernatants were transferred to Zeba spin desalting columns (40 k MWCO, Thermo Sci, IL, USA). Then, three freeze-thaw cycles were applied, and the activity was assessed by following disappearance of sepiapterin at 420 nm in a medium containing 50 mM Tris-HCL buffer pH 7.5, 0.1% Triton X-100, 200 µM sepiapterin, 600 µM NADPH and 25 µg/mL extract protein. The extinction coefficient of $\epsilon 420 = 10.4\ mM–1.\ cm–1$ was used for the calculations after correction for multiwell plates. Activities are depicted as U / mg protein, and 1 U corresponds to 1 µmol consumed NADPH / min at 37 °C.

2.13. Gene expression analysis by quantitative real-time PCR

Total RNA was isolated from hippocampal samples from mice treated with BH4 (4 pmol; i.c.v.; 24 h, by using the TRIzol®/chloroform/isopropanol method. The quantity and purity of extracted RNA was estimated by using the spectrophotometer apparatus NanoDrop, at 260 nm and 280 nm. The cDNA was synthesized by reverse transcription kit “M-MLV Reverse Transcriptase” (Sigma; St. Louis, MO, USA), according to the instructions recommended by the manufacturer. qPCR was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and specific primers (Table 1) for each gene. The primers were designed using the “BLAST” available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi according exons specific for each protein. Reactions were performed in the ABI PRISM 7900HT equipment (Applied Biosystems, Foster City, USA) in the Multiuser Laboratory for Biological Studies (LAMEB, UFSC, Brazil). The results were analyzed using Sequence Detection Systems software (SDS) version 2.4. The critical comparative threshold method $2^{-\Delta Ct}$ was used to calculate the relative number of transcripts in the samples. In this method, the average Ct gene of interest is subtracted from the average Ct internal control (β-actin), resulting in a ΔCt. To calculate gene expression, the ΔCt value obtained is replaced in $2^{-\Delta Ct}$ formula. The obtained final numbers are presented as the ratio between the expressions of the gene of interest relative to the internal control gene. The results were expressed as mean ± SEM of three independent animals performed in triplicate.

2.14. Protein determination

The protein contents were determined by the Lowry method [23], using bovine serum albumin as a standard.

2.15. Statistical analysis

All data are presented as mean ± SEM. *Student t-test* and one-way analysis of variance followed by the *post hoc* Duncan multiple range test were applied for means comparisons when data had a normal distribution (Fig. 1 and 2B, C and D). Data from the inhibitory avoidance task are presented as median and interquartile ranges, and comparisons among groups at the conditioning and test session were performed using the Kruskal-Wallis test, since the data generated was non-continuous. The data from electrophysiological experiments were expressed as mean ± SEM and analyzed by the one-way analysis of variance (ANOVA) with repeated measures, followed by the *post hoc* Student–Newman–Keuls test, because the parameter needs to be tested over time. Effect sizes were calculated according to Cohen [24].

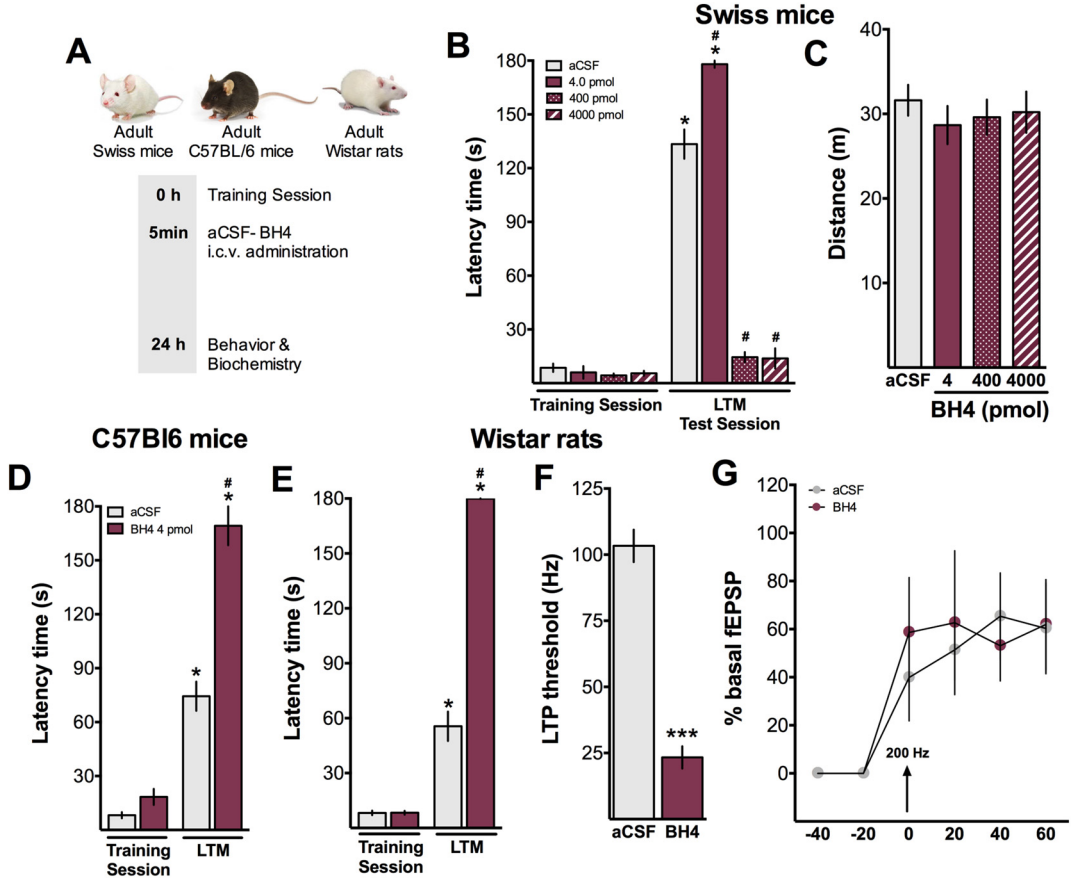

Fig. 2. Tetrahydrobiopterin (BH4) administration enhances hippocampal aversive learning. BH4 was injected intracerebroventricularly (i.c.v.) and behavior and biochemistry were assessed 24 h later (A). Adult Swiss mice were subjected to the training section in the step-down avoidance task, immediately after receiving a single i.c.v. injection of BH4 (4, 400 and 4000 pmol; 1 μL) and 24 h later were subjected to the test session. The long-term memory (LTM) was evaluated as the latency time (s) in the step-down avoidance task (B) and the spontaneous locomotor activity as the distance traveled (m) in the open-field test (C). Adult C57BL/6 J mice were subjected to the same experimental protocol, receiving only the 4 pmol dose of BH4. The LTM was measure 24 later (D) as described in C. Adult Wistar rats were also subjected to the same experimental conditions as C57BL/6 J and 24 h later the LTM was assessed (E) and the hippocampus sliced for electrophysiological analyses to measure the LTP threshold (Hz) (F) and the percentage of basal field excitatory postsynaptic potential (fEPSP; G). Data from the inhibitory avoidance task are presented as median and interquartile ranges. *P < 0.05, ***P < 0.001 vs aCSF; #P < 0.05 vs BH4 (Kruskal-Wallis test). All other values are presented as mean ± SEM. Data from LTP threshold was analyzed by Student t-test for unpaired samples. *P < 0.05 vs aCSF. The data from fEPSP were analyzed by the one-way analysis of variance (ANOVA) with repeated measures, followed by the *post hoc* Student Newman Keuls test.

Differences between groups were rated significant at $P \leq .05$. The statistical analyses were performed using IBM® SPSS Statistics® software (version 21) and graphs were prepared using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. BH4 administration increases the activity of the hippocampal salvage pathway

Fig. 1B-J shows the effect of BH4 administration (i.c.v.; 4 pmol; 1 μL; 24 h) on the activity of the BH4 salvage pathway in the mouse hippocampus. The percentage of intact BH4 before injections was about 20%, when compared to a solution prepared in HCl, indicating that most of the BH4 solution injected in the ventricles was already transformed into BH2 (Fig. 1B). BH4 administration provoked increased levels of BH4 (Fig. 1C) [$t_{(8)} = 1.95$; $P < 0.05$; $d = -1.40$; $n = 5$/group], and increased SPR activity (Fig. 1D) [$t_{(6)} = 1.86$; $P < 0.05$ $d = -0.86$; $n = 5$/group] in the mouse hippocampus 24 h after the injections, suggesting the activation of the BH4 salvage pathway (Fig. 1E). In agreement, DHFR gene expression, an enzyme proposed to recycle BH2 into BH4 in the BH4 salvage pathway, was also increased in the hippocampus of mice receiving BH4 (Fig. 1G) [$t_{(6)} = 1.86$; $P < .005$ $d = -0.97$; $n = 5$–6/group], while the levels of the other BH4 synthesizing enzymes, namely GTPCH (rate-limiting enzyme of the *de novo* pathway), PTP (*de novo* pathway), and DHPR (main enzyme from the recycling pathway), remained unchanged. Therefore, the injection of BH4 (about 80% oxidized BH4) increased the synthesis of BH4 in the hippocampus, possibly by stimulating the BH4 salvage pathway.

3.2. BH4 administration enhances hippocampal aversive learning in adult rodents

Fig. 2 shows the effect of a single i.c.v. BH4 injection on aversive memory in various rodent species and strains. Fig. 2B shows that BH4 (4 pmol; i.c.v.) administered immediately after the training session significantly increased the latency to step-down in the inhibitory avoidance task at the 24 h test session in adult Swiss mice. The figure also shows that the higher doses of BH4 (400 and 4000 pmol) compromised the LTM formation [$H_{(3)} = 17.54$; $P < 0.001$; $d = -2.21$; $n = 7$/group]. Fig. 2C shows that the higher latencies to step-down were not due to motor impairment in the Swiss mice. Similarly, 4 pmol of BH4 solution enhanced LTM retention in C57BL/6 J mice [$U = 15.00$; $P < 0.05$; $d = 1.08$; $n = 5$/group] and Wistar rats (Fig. 2D-E) [$U = 45.00$; $P < .001$; $d = 1.74$; $n = 9$/group]. The

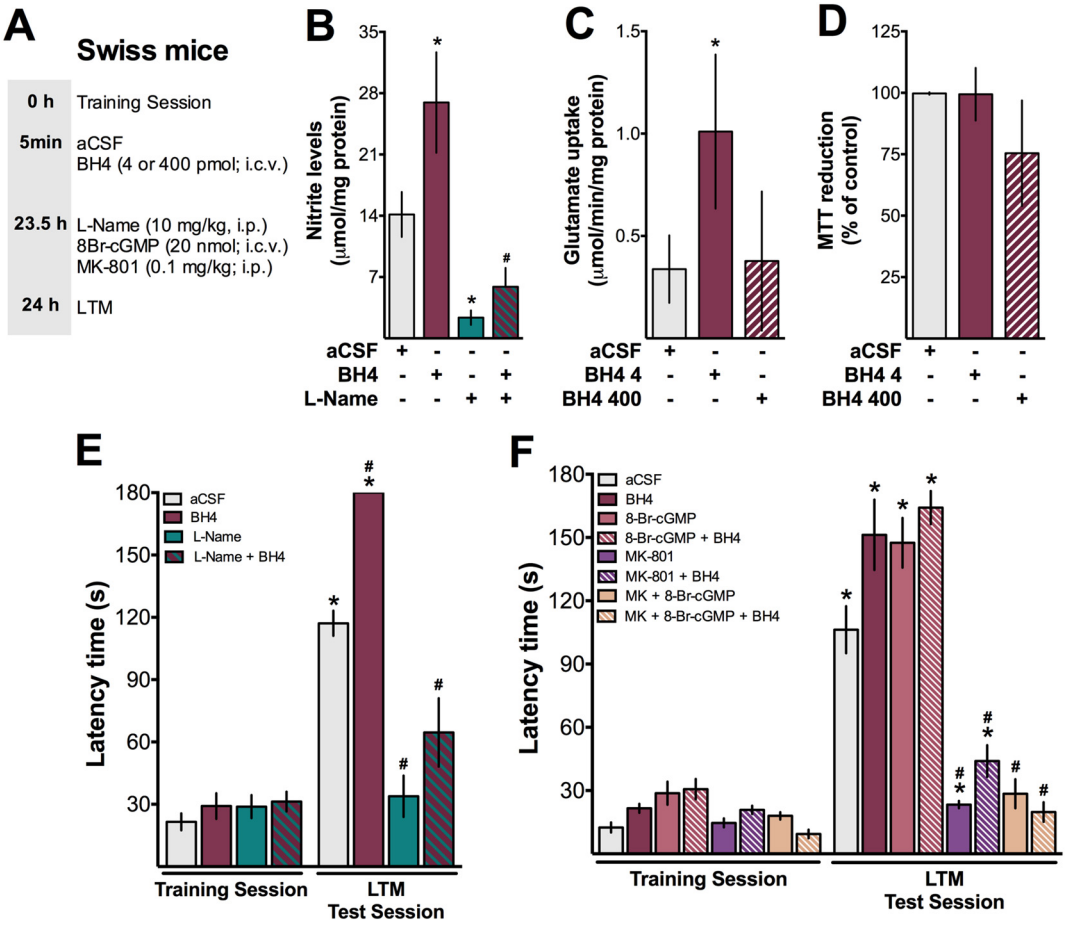

Fig. 3. Tetrahydrobiopterin (BH4) facilitates aversive learning by stimulating glutamatergic neurotransmission. BH4 was injected intracerebroventricularly (i.c.v.) and behavior and biochemistry were assessed 24 h later. In some experiments L-Name (nitric oxide synthase inhibitor), MK-801 (NMDA receptor antagonist) and 8-Br-cGMP (protein kinase G activator) were administered (i.p.) 30 min prior to the behavioral assessment (A). Adult Swiss mice received a single i.c.v. injection of BH4 (4 or 400 pmol) and 24 h later the hippocampus was dissected, sliced and used for biochemical analyses. Nitrite levels were analyzed by the Griess method (A), glutamate uptake by scintillation counting (B) and cell viability (C) by MTT reduction. Swiss mice were subjected to the training section in the step-down avoidance task, and immediately after received a single i.c.v. injection of BH4 (4 pmol). The animals received an i.p. injection of two inhibitors, L-NAME (10 mg/kg) and MK-801 (0.1 mg/kg), and an i.c.v. administration of 8-bromo-cGMP (20 nmol) 30 min before the test session. Long-term memory (LTM) was evaluated as the latency time (s) to step-down in the avoidance task (E and F). Data from the inhibitory avoidance task are presented as median and interquartile ranges and analyzed by the Kruskal-Wallis test, *P < 0.05 vs aCSF; #P < 0.05 vs BH4. Pair comparisons were performed by the Mann-Whitney test, *P < 0.05 vs aCSF; #P < 0.05 vs BH4. All other values are presented as mean ± SEM and analyzed by one-way analysis of variance (ANOVA) followed Tuckey's *post hoc* test.

facilitated aversive memory observed in rats was associated by a reduced threshold to generate LTP in rats (Fig. 2F) [$t_{(10)} = 10.73$; $P < 0.001$; $d = 5.74$; $n = 6$/group]. However, no differences were found in the magnitude of fEPSP (field excitatory postsynaptic potential) under BH4 treatment (Fig. 2G). Therefore, higher hippocampal levels of BH4 enhanced aversive memory and reduced the threshold to generate LTP.

3.3. BH4 administration facilitates aversive learning by stimulating the glutamatergic neurotransmission

Fig. 3 shows the participation of the NO/glutamatergic neurotransmission on the BH4-induced enhanced cognition in Swiss mice. Considering that BH4 is a mandatory cofactor for NO synthesis, and that NO probably acts as a retrograde neurotransmitter to favor the LTP process, Swiss mice were treated with L-Name (10 mg/kg; i.p.; Fig. 3A) to block LTM. Fig. 3B shows that L-Name blocked NO production, and that co-treatment with BH4 slightly rescued NO levels (BH4 effect: [$F_{(1,16)} = 35.15$; $P < 0.001$], L-Name effect: [$F_{(1,16)} = 142.3$; $P < 0.001$], interaction [$F_{(1,16)} = 11.34$; $P < 0.001$]; $d = 0.83$; $n = 7$/group). As a retrograde molecule, NO-linked LTP dependence relies on the N-methyl-D-aspartate (NMDA)-type glutamate receptor activity [25]. Thus, as a measure of the glutamatergic activity, we assessed glutamate uptake by hippocampal slices, 24 h after administering BH4. Fig. 3C shows that BH4 at the 4 pmol dose significantly increased glutamate uptake, while 400 pmol did not modify that parameter [$F_{(2,10)} = 6.75$; $P < 0.05$; $d = 0.57$; $n = 4$–5/group]. The higher glutamate uptake induced by BH4 did not compromise the viability of hippocampal slices (Fig. 3D). To better understand the participation of the NO/glutamatergic neurotransmission, we investigated the cognitive enhancer effect of BH4 in the presence of the following: L-Name (an inhibitor of all isoforms of NOS), cell permeant 8-Br-cGMP (NO-dependent second messenger, activator of PKG), and MK-801 (non-competitive NMDA receptor antagonist). Fig. 3E shows that the administration of L-Name (10 mg/kg; i.p.) inhibited the enhancing effect of BH4 on LTM, which was not recovered by BH4 co-treatment (LTM session: [$H_{(3)} = 22.36$; $P < 0.001$; $d = 1.44$; $n = 7$/group];). Similarly, Fig. 3F shows that by blocking glutamatergic neurotransmission with MK-801, the positive effect of BH4 on memory was markedly reduced. However, 8-Br-cGMP treatment did not compromise BH4's effect, nor did it rescue the amnesic effect induced by MK-801 (LTM session: [$H_{(7)} = 39.13$; $P < 0.001$]; $d = 3.54$; $n = 4$–15/group).

4. Discussion

BH4 is essential for diverse processes and is ubiquitously present in all tissues of higher organisms. The best-investigated function of BH4 is its action as a natural cofactor of the aromatic amino acid hydroxylases, phenylalanine-4-hydroxylase, tyrosine-3-hydroxylase and tryptophan-5-hydroxylase, as well as of all three forms of nitric oxide synthase (for a review see [5]). However, the participation of BH4 in other biological processes is not well understood or has not been identified yet. The fine regulation of BH4 intracellular concentration is the result of a tuned balance of three biosynthetic pathways: the *de novo*, the recycling and the recently identified salvage pathway [3]. Thus, the strict regulation of the BH4 synthesis points out more cellular/physiological processes might be dependent on its active synthesis.

Our group has recently demonstrated that excessive BH4 levels produced by damaged sensory neurons, nerve tissues and infiltrating macrophages produce hypersensitivity to pain in inflammatory and neuropathic pain models [8]. Central sensitization underlies many pain conditions ranging from nerve injury-induced allodynia to headache. This phenomena described for the first time by Woolf [13] shares cellular and molecular mechanisms with learning and memory. Sandkuhler and coworkers [14] have demonstrated that the neurophysiological basis for central sensitization involves the generation of long-term potentiation (LTP). This LTP is similar to that observed in the hippocampus, except that in a subset of neurons this LTP can be evoked by low frequency stimulation (generally LTP is evoked only by high frequency stimulation). Since, in both cases, the glutamate neurotransmission, which requires NO synthesis, is stimulated, we hypothesized that BH4 might be inducing pain hypersensivity by stimulating the generation of LTP. If that is the case, BH4 might also enhance hippocampal-mediated cognition.

Data presented here showed that a single i.c.v. injection of BH4 enhances learning and memory in various species and strains of rodents. To our knowledge, this is the first evidence showing that this endogenous enzyme cofactor enhances cognition. In addition, the molecular mechanism involved appears to be linked to the activation of glutamatergic neurotransmission and cell threshold reduction to trigger LTP.

A well-established protocol to assess learning and memory in rodents is the one-trial step-down test, where animals learn to suppress the exploratory tendency to avoid aversive stimuli [26]. Moreover, the training session in the step-down test induces LTP generation in the hippocampus [26–28], and it has been demonstrated to be essential for LTM formation. LTP represents the acquisition and maintenance of memories at a synaptic level [26–29]. Our observations showed that BH4, when administered immediately after the training session (after acquisition, and before consolidation), increased the latency to step-down, probably as a result of facilitated learning in the inhibitory avoidance task. The reduced threshold to generate LTP, following i.c.v. BH4, further supports this interpretation, indicating that this pteridine enhances hippocampal synaptic plasticity and that it is unlikely to involve impairments of locomotion. Interestingly, it has been reported that BH4 administration in patients affected by phenylketonuria improved working memory and brain activation [30].

LTP induction is typically dependent on the activation of NMDAR (*N*-methyl-D-aspartate receptor) [29], and downstream intracellular signaling cascades, including NO synthesis, that ultimately are responsible for altered synaptic efficacy. Moreover, the induction and maintenance of LTP requires that the retrograde messenger NO, released from postsynaptic cells acts on presynaptic terminals, where it enhances neurotransmitter release (for a review see [31]). The key NO participation in neuroplasticity and cognition has been demonstrated by the use of NOS inhibitors and NO scavengers, where LTP is blocked or abolished [32]. NO signals LTP through cGMP synthesis and cGMP-dependent protein kinase activation. This signaling is responsible for upregulating transcription and translation changes in postsynaptic cells, and the inhibition of the pathway also compromise memory and learning [33]. In this context, we demonstrated here that the administration of the potent NOS inhibitor, L-NAME, and of the NMDAR antagonist, MK-801 (opened-channel blocker), in Swiss mice impaired the cognitive enhancement induced by BH4 on LTM, even when the cGMP analogue (8-bromo-cGMP) and/or BH4 were co-administered, suggesting the involvement of common mechanisms. Furthermore, reduced hippocampal NO levels showed impaired performance in the step-down avoidance task, when L-Name was administered. These results support the hypothesis that BH4 effects on the memory is dependent of NO signaling, as well as NMDAR activation. On the other hand, when BH4 was administered, increased levels of NO were generated. High NO concentrations are known to be neurotoxic [34], and excessive activation of NMDAR can also induce glutamatergic excitotoxicity (Waring et al., 2005). However, no cell damage was observed, since the BH4 injection did not change hippocampal viability.

The synthesis of NO requires as enzyme cofactors, oxygen, NADPH and BH4. BH4 is always the limiting factor in the reaction, controlling therefore the levels of NO. When BH4 is oxidized or the synthesis is compromised, NOS is uncoupled and toxic reactive species are generated [35]. Taking into account the essential role of BH4 in the synthesis of NO, we suggest that the molecular mechanism involved in BH4 memory effects is dependent on NO signaling. In addition, BH4 at the physiological levels has been reported to have antioxidant properties and favor mitochondrial activity, mainly during oxidative stress [36]. Under these conditions for example, BH4 will be oxidized to BH2, and therefore, NOS uncoupling will be induced [37], and consequently memory will be impaired [38]. Thus, BH4 concentration is a critical factor in determining its protective or deleterious role.

On the other hand, BH4 in solution at neutral pH (*i.e.* aCSF; pH) is easily oxidized and auto-oxidation of BH4 in solution has previously been described. BH4 in solution at pH 6.8 or 7.6 generates an ultraviolet spectrum identical to that of authentic BH2 [39, 40]. We observed here that after BH4 solution was prepared for i.c.v. injections, a spontaneous oxidation occurred (about 80% of oxidation). However, levels of BH4 were significantly increased in the hippocampus, pointing out that a metabolic pathway was activated in order to recycle BH4. In agreement, the expression of DHFR, an enzyme proposed to recycle BH2 into BH4 in the BH4 salvage pathway, was exclusively increased in rodents receiving BH4. In addition, SPR activity was also increased, suggesting that the BH4 salvage pathway is active in the rodent brain. Therefore, this mechanism might be activated in patients orally treated with synthetic BH4, which may go under auto-oxidation during the absorption process, but once it reaches the brain it is reduced back to BH4. Altogether, it can be suggested that endogenous BH4 and the oral administration of the compound might enhance memory and learning.

Finally, we also observed here that high amounts of BH4 markedly compromised LTM formation. These data are in agreement with Kim and coworkers [41]. After injecting 1 μmol BH4 i.c.v. in rats, researchers observed brain toxicity shown by degeneration of the dopaminergic terminals in the striatum. They thus proposed that high levels of BH4 might participate in the physiopathology of Parkinson's disease [41]. Extrapolating the results brought in our present work, we can speculate that high levels of BH4 induced for example under inflammatory conditions (BH4 is a mandatory cofactor for nitric oxide synthase type II; isoform induced by inflammation), will compromise cognition probably by inducing oxidative stress, two conditions observed in neurogenerative diseases, including Parkinson's disease.

5. Conclusions

To our knowledge, this is the first evidence showing that BH4 enhances memory by activating the glutamatergic neurotransmission and the retrograde activity of NO. In addition, it was also demonstrated here that BH2 can be converted into BH4 by activating the BH4 salvage pathway under physiological conditions. Altogether, it can be suggested

that the modulation of the BH4 pathway might enhance memory and learning.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

The authors are grateful to Ted Griswold for language editing. This work was supported by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil, 479222/2013-4), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil, PVE: 004_2013 and 88881.062164/2014-01, INCT 189405 16/2014), FAPESC/CNPq (Programa de Apoio a Núcleos de Excelência PRONEX; NENASC Project), SECyT/UNC (Secretaría de Ciencia y Técnica de la Argentina, CONICET Nº PIP 11220090100630, Cat. A 2012-2013 Res. 162/12) , FONCyT (Fondo para la Investigación Científica y Tecnológica, Argentina PICT 2011-2476) and CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina). Latini A, Prediger RD and Aguiar Jr., AS are CNPq fellows.

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