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Neopterin acts as an endogenous cognitive enhancer

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ABSTRACT

Neopterin is found at increased levels in biological fluids from individuals with inflammatory disorders. The biological role of this pteridine remains undefined; however, due to its capacity to increase hemeoxygenase-1 content, it has been proposed as a protective agent during cellular stress. Therefore, we investigated the effects of neopterin on motor, emotional and memory functions. To address this question, neopterin (0.4 and/or 4 pmol) was injected intracerebroventricularly before or after the training sessions of step-down inhibitory avoidance and fear conditioning tasks, respectively. Memory-related behaviors were assessed in Swiss and C57BL/6 mice, as well as in Wistar rats. Moreover, the putative effects of neopterin on motor and anxiety-related parameters were addressed in the open field and elevated plus-maze tasks. The effects of neopterin on cognitive performance were also investigated after intraperitoneal lipopolysaccharide (LPS) administration (0.33 mg/kg) in interleukin-10 knockout mice (IL-10$^{-/-}$). It was consistently observed across rodent species that neopterin facilitated aversive memory acquisition by increasing the latency to step-down in the inhibitory avoidance task. This effect was related to a reduced threshold to generate the hippocampal long-term potentiation (LTP) process, and reduced IL-6 brain levels after the LPS challenge. However, neopterin administration after acquisition did not alter the consolidation of fear memories, neither motor nor anxiety-related parameters. Altogether, neopterin facilitated cognitive processes, probably by inducing an antioxidant/anti-inflammatory state, and by facilitating LTP generation. To our knowledge, this is the first evidence showing the cognitive enhancer property of neopterin.

1. Introduction

Neopterin is a byproduct of the tetrahydrobiopterin (BH4) *de novo* biosynthetic pathway. Increased levels of this metabolite in biological fluids have been used for decades as a sensitive biomarker of cell-mediated immune activation (Fuchs et al., 1989; Widner et al., 2002), *i.e.* in bacterial and viral infections, as well as in neurodegenerative, neurologic and cardiovascular diseases (Bechter et al., 2010; Denz et al., 1990; Firoz et al., 2015; Hagberg et al., 2010; Parker et al., 2013; Sucher et al., 2010). Physiological neopterin values have been reported to be about 5–8 nmol/L in the blood and similar levels in the cerebrospinal fluid (CSF) (Hagberg et al., 1993).

Neopterin production in humans has traditionally been linked to monocytic cells under interferon-γ (IFN-γ) or interleukin-1β (IL-1β) stimulation, among others (Franscini et al., 2003). These stimuli trigger the first rate-limiting step enzyme in the BH4 *de novo* pathway, the guanosine triphosphate cyclohydrolase I (GTPCH). In this pathway, BH4 is generated from guanosine triphosphate (GTP), which is converted to 7,8-dihydroneopterin triphosphate by GTPCH (Blau et al., 1989; Millner et al., 1998; Werner et al., 1990). Then, 6-pyruvoyl tetrahydropterin synthase (PTPS) removes the phosphates to generate 6-pyruvoyl-

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tetrahydropterin, which is further converted to BH4 by sepiapterin reductase (SR) (Werner et al., 1990). Under stimulation, GTPCH activity is increased up to 100-fold, while PTPS and SR remain slightly increased (Kerler et al., 1989; Werner et al., 1990; Werner-Felmayer et al., 1993a). Consequently, in these conditions, PTPS becomes the rate-limiting enzyme in the pathway favoring neopterin formation (Werner et al., 1990; Werner-Felmayer et al., 1993b).

Neopterin production by the central nervous system (CNS) has recently been proposed by Kuehne et al. (2013) based on observations that neopterin concentrations in cerebrospinal fluid were higher than those in serum from patients suffering from schizophrenic spectrum disorders with no blood-brain barrier dysfunction. In addition, our group demonstrated that in both rat striatal astrocytes and hippocampal slices neopterin was synthesized and release under cellular stress conditions (Ghisoni and Latini, 2015). However, the exact physiological role of the biology of this pteridine, even in the periphery is virtually unknown. Some reports have associated micro or millimolar concentrations of extracellular neopterin with oxidant activity (Wede et al., 1999), nuclear factor kappa B (NF-κB) up-regulation (Cirillo et al., 2006; Hoffmann et al., 1999, 1996), or cell death (Baier-Bitterlich et al., 1995).

Recent studies from our group using the administration of pico or namolar concentrations of neopterin have shown clear and distinct effects. The single intracerebroventricular (i.c.v.) administration of neopterin promoted resistance to oxidative stress in mouse brain tissue (Ghisoni and Latini, 2015), and the astrocyte exposure to neopterin resulted in increased immunocontent of hemeoxygenase-1 (HO-1) (Ghisoni et al., 2015), an early biomarker of the antioxidant and cytoprotective Nrf2/ARE pathway activation. The relationship of this endogenous pteridine with the Nrf2 pathway has also been addressed by McNeill et al. (2015), showing that BH4 deficient mice had reduced expression of Nrf2. In addition, activators of this pathway are receiving growing attention for the treatment of oxidative stress-related disorders, including neurological and neurodegenerative diseases, where cognitive impairments are observed; and also for the enhancement of age-associated cognitive decline (Cutuli et al., 2014; Liu et al., 2015a; Yu et al., 2015). Therefore, herein we investigated the potential role of neopterin on learning and memory processes in adult and aged rodents, under basal and inflammatory conditions, based on its Nrf2 activation-linked activity.

2. Material and methods

2.1. Animals

In order to study the effect of neopterin on cognition through different age and rodents strain and species, the following experimental animals were used in the present investigation. Experiments were conducted using male Swiss albino (60 day-old), C57BL/6 and IL-10 knockout (IL-$10^{(-/-)}$) mice (17 month-old) from the Central Animal House of the Centre for Biological Sciences, Universidade Federal de Santa Catarina (Florianópolis, Brazil) and adult male Wistar rats (60–90 day-old), obtained from the Central Animal House of the Pharmacology Department of School of Chemical Sciences, Universidad Nacional de Córdoba (Córdoba, Argentina). As genetic background controls for IL-$10^{(-/-)}$ mice, C57BL/6 mice were used in the experiments. Some C57BL/6 mice were used at INSERM UMR S894, Centre de Psychiatrie et Neurosciences (Paris, France). All the animals were kept under standard laboratory conditions (12 h light-dark cycle, lights on at 07:00 h, temperature 21 ± 1 °C) with free access to food and water. The experimental protocols were approved by the Ethics Committee for Animal Research (PP00425/CEUA) from the Universidade Federal de Santa Catarina, from the Universidad Nacional de Córdoba (RES-48-2015), and from the French Minister of Research (Protocol authorization #00966.02, Directive 2010/63/UE, 22/09/2010) and followed the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989. All efforts were made to minimize the number of animals used and their suffering.

2.2. Intracerebroventricular administration of neopterin

For mice: free-hand transcranial injections were made for local injections in ventricle (i.c.v.); the relative coordinates were used according to the atlas of Paxinos and Franklin (2001). The coordinates relative to Bregma were anterior: −0.5 mm; lateral: ±1.0 mm; vertical: −2.0 mm for the left ventricle. Animals were injected with neopterin or vehicle (artificial cerebrospinal fluid; aCSF) under isoflurane anesthesia (2–3%; 1L/min) in C57BL/6 mice or diethyl ether (1.9% in a cotton ball inside a conical tube or small chamber) in Swiss and IL-$10^{(-/-)}$ mice by using a Hamilton syringe connected by Pe-10 polyethylene tubing. The delivered volume was 1 μL over one-minute period. The i.c.v. injected dose in mice (0.4 pmol) was chosen in order to elicit a transient increase of CSF neopterin of up to 50 nM. For the calculations, a 35 μL was considered as the maximum CSF volume (Pardridge, 1991).

For rats: animals were cannulated in the lateral ventricle area with steel guide cannula, according to an atlas (Paxinos and Watson 1982). The coordinates relative to Bregma were anterior: −0.8 mm; lateral: ±1.5 mm; vertical: −4.0 mm for left ventricle. Cannulas were fixed to the skull surface with dental acrylic cement. Animals were under ketamine hydrochloride (55 mg/kg) and xylazine hydrochloride (11 mg/kg) anesthesia during the whole procedure. Rats were allowed to recover from surgery during seven days and were handled daily to habituate them to the injection procedures. After the recovery period, animals were injected with neopterin or vehicle (aCSF) by using a Hamilton syringe connected by Pe-10 polyethylene tubing, without anesthetization. The delivered volume was 1 μL over one-minute period. The injected dose of neopterin in rats (4 pmol) was ten times higher than in mice, since rats have higher CSF volume (Pardridge, 1991).

2.3. LPS Treatment

Mice were injected intraperitoneally (i.p.) with saline or Escherichia coli LPS (lot 3129, serotype 0127:B8, Sigma; 0.33 mg/kg; (Henry et al., 2009).

2.4. Behavior

2.4.1. Open field

The locomotor activity was evaluated in the open field arena. The open field was made of wood covered with impermeable Formica (100 cm × 100 cm × 50 cm) and the experiments were performed in a sound-attenuated room under low-intensity light. Each animal was placed in the center of the apparatus and the exploratory activity was registered for 5 min (Hall and Ballachey, 1932).

2.4.2. Step-down inhibitory avoidance

Short- and long-term memories were evaluated in the inhibitory avoidance task using a previously described protocol (Roesler et al., 1999). The inhibitory avoidance apparatus was an acrylic box (50 cm × 25 cm × 25 cm), which floor consisted of parallel stainless-steel bars (1 mm diameter) spaced 1 cm apart. A platform (7-cm wide 2.5-cm high) was placed on the floor against the left wall. 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 0.4-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 1.5 h and 24 h after a single training session to evaluate short- and long-term memories, respectively.

2.4.3. Elevated plus-maze

The plus maze apparatus was made of wood and consisted of two open arms measuring 50 × 10 cm, and two enclosed arms of 50 × 10 cm, 40 cm-high walls enclosing the latter. The arms extended from a central platform (10 × 10 cm); the whole apparatus was elevated 50 cm above the floor. To prevent rats from falling down, the open arms were bordered by transparent plastic 1 cm high. The testing was performed under red light. Individual rats were placed onto the central platform and observed for 5 min. The behavioral performances recorded were: the number of entries in the open arms; the number of entries into closed arms; time spent in open arms; rearing, grooming and risk-assessment. The test was performed as a single trial per animal (Pellow et al., 1985).

2.4.4. Fear conditioning

The fear conditioning paradigm was done according to Mongeau et al. (2007) protocol with modifications. The test was conducted in a chamber (length 26 cm × wide 18 cm × high 22 cm), housed in a sound-attenuating box, possessing aluminum sidewalls and Plexiglas rear and front walls, and a stainless steel grid floor (MED Associates, St. Albans, VT). On day-1, the mouse was placed into the chamber washed with a vanilla odor and allowed to acclimate for 3 min. Then, the animal was exposed to six episodes of a tone (conditioned stimulus, CS) of 2500 Hz frequency and 85 dB intensity for 30 s immediately followed by a foot shock of 0.75 mA for 2 s (unconditioned stimulus, US). The interval between the tone + shock pairings was 2 min (inter-trial interval, ITI). On day-2 (24 h later), mice were exposed to the same procedure as on day-1, but without CS or US and allowed to explore for 20 min. On day-3 mice were tested for cued fear memory by returning to the chamber with various modifications (no vanilla odor, addition of hatch designs to the walls and lights on). Only the CS was presented 40 times separated by an ITI of 5 s. All sessions were recorded using infrared cameras and controlled by a computerized system interface (MED Associates, St. Albans, VT). Freezing behavior, defined as complete absence of voluntary movements except for respiration, was measured manually in the ANY-maze software (Stoelting) and expressed as percentage of time spent freezing.

2.5. Electrophysiology

Electrophysiological experiments were carried out using an *in vitro* hippocampal slice preparation (Perez et al., 2010). Immediately after the step-down test, rats were killed between 11.00 am and noon to prevent variations caused by circadian rhythms or nonspecific stressors (Teyler and DiScenna, 1987). Briefly, the hippocampal formation was dissected, and transverse slices of approximately 400 μm thick were placed in a recording chamber (BSC-BU Harvard Apparatus), perfused with a standard Krebs solution (124.3 mM NaCl, 4.9 mM KCl, 1.3 mM $MgSO_47H_2O$, 1.25 mM H_2KPO_4, 25.6 mM $HNaCO_3$, 10.4 mM glucose, and 2.3 mM $CaCl_22$-H_2O) saturated with 95% O_2 and 5% CO_2. The rate of perfusion was 1.6 mL/min, and the bathing solution temperature was kept at 28 °C for whole the duration of the experiment using a temperature controller (TC-202A Harvard Apparatus). A stimulating electrode made of two twisted wires, insulated except for the cut ends (diameters 50 μm), was placed in the perforant path and the recording electrode, a glass micropipette (10–20 μm tip) was inserted in the dentate granule cell body layer. Only slices showing a stable response were included in this study. Field excitatory post-synaptic potentials (fEPSP) that responded to 0.2 Hz stimuli were sampled twice, during 4 s, each 5 min, within a 40 min period, until fEPSP stabilization (baseline). Once no further changes were observed in the fEPSP amplitude, one of the two stimulation protocols were applied. In the first protocol, LTP was generated using the classical tetanization paradigm consisting of three 100-Hz high-frequency stimulation (HFS) trains (of 1 s duration each) given at 20 s intervals, delivered by an A310 accupulser pulse generator (World Precision Instruments Inc.). LTP was considered to have occurred when the fEPSP amplitude recorded after the stimulus, at 0.2 Hz, had risen at least 30% from baseline and persisted for 60 min. The second protocol, involved a tetanization paradigm consisting of three 5-Hz frequency stimulation trains (of 1 s duration each) given at 20 s intervals. LTP was considered to have occurred as described above. All collected data were recorded and stored for future analysis.

2.6. Cytokine measurement

Mouse brain levels of IL-1β and IL-6 were determined by ELISA (R&D Systems, MN). In brief, mice were euthanized, the brain tissue samples were immediately dissected, weighed, frozen in liquid nitrogen, without any previous perfusion, and stored at −80 °C until the assay. For cytokine assays, samples were homogenized in phosphate-buffered saline (pH 7.4) containing Tween 20 (0.05 %), 0.1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 2 ng/mL aprotinin, and 0.1 mM benzemethonium chloride. Tissues were homogenized (1:10), centrifuged (3000×*g* for 10 min at 4 °C), and the supernatant obtained were used for analysis. The levels of IL-1β and IL-6 were measured using sample aliquots of 100 μL and mouse cytokine ELISA kits from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's instructions. The levels of cytokines were estimated by interpolation from a standard curve by colorimetric measurements at 450 nm (correction wavelength 540 nm) on an ELISA plate reader (Infinite 200 PRO TECAN Switzerland). Assays were sensitive to 1.5 pg/mL of IL-1β and 1.8 pg/mL of IL-6, inter- and intra-assay coefficients of variation being less than 10%. All results were expressed as picograms per milligram of protein.

2.7. Protein determination

Brain homogenate protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

2.8. Statistics

Data from the inhibitory avoidance task are presented as median and interquartile ranges, and comparisons among groups were performed using Mann–Whitney *U* tests. The data from electrophysiological experiments were expressed as mean ± S.E.M. and analyzed by the one-way analysis of variance (ANOVA) with repeated measures, followed by the *post hoc* Student–Newman–Keuls test. Data from open-field and elevated plus-maze tests were analyzed using the Student *t* test for unpaired samples. Data from biochemical measures are expressed as mean ± SEM and were analyzed using two-way ANOVA followed by the *post hoc* Duncan multiple range test. Differences between groups were rated significant at $P \leqslant 0.05$. The statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Neopterin facilitates aversive learning in adult Swiss mice

Fig. 1A–C shows the effect of a single i.c.v. administration of neopterin (0.4 or 4 pmol) in Swiss mice, following the experimental design shown in Fig. 1A. Fig. 1B shows that the 0.4 pmol dose of neopterin significantly increased the latency to step-down in the inhibitory avoidance task at the 1.5 h and 24 h test sessions, from the acquisition trial (training session) (STM = [H(2, N = 30) = 9.83; $P < 0.05$]; LTM = [H(2, N = 30) = 8.79; $P < 0.05$). The figure also shows that the 4 pmol dose did not induce changes in short- (1.5 h; STM) or long-term (24 h; LTM) memories. Fig. 1C shows that the effects of neopterin on the animals' performance of the step-down inhibitory avoidance task were not related to spontaneous locomotion deficits, since the total distance traveled and the mean speed in the open field test were not altered by neopterin.

3.2. Neopterin enhances cognition by facilitating hippocampal LTP generation in adult Wistar rats

Next, was investigated the neopterin effect on cognition in rats. The tested dose was 4 pmol since rats have 10 times more CSF volume than mice. Fig. 2A-E shows the effect of a single i.c.v. administration of neopterin (4 pmol) in Wistar rats, following the experimental design shown in Fig. 2A. Fig. 2B shows that 4 pmol dose of neopterin significantly facilitated long-term memory retention (24 h; LTM) in the inhibitory avoidance test, compared to the acquisition trial (training session) [$F_{(3,14)} = 12{,}09$; $P < 0{,}05$]. Moreover, this effect was not related to neopterin-induced alterations in anxiety-related and spontaneous locomotor activity, since the percentage of time spent by the animals in the open arms and the number of entries in the closed arms of the elevated plus-maze test did not differ from controls (vehicle; Fig. 2C and D, respectively). Fig. 2E and F shows that the effect of neopterin on cognition was accompanied by a reduced threshold to generate LTP. Neopterin generated at stimuli as low as 5 Hz, LTP of the same magnitude as those observed in control conditions with the 100 Hz intensity stimulation. Once LTP was generated, no differences were found in the magnitude of *fEPSP* amplitude between groups (% of *fEPSP* amplitude increase from baseline) (Fig. 2F).

3.3. The cognitive enhancing properties of neopterin are also linked to reduced IL-6 production in aged IL-10 null mice

In order to study the effect of neopterin on cognition in aged mice with an excessive inflammatory response, the following experimental design used IL-10 null mice of 17 months of age. Fig. 3A–F shows the effect of a single i.c.v. administration of neopterin (0.4 pmol) in LPS-treated IL-$10^{-/-}$ aged mice, following the experimental design shown in Fig. 3A. Fig. 3B shows that LPS administration (0.33 mg/kg; i.p.) impaired spontaneous locomotor activity in mice, depicted by a significant reduction of travelled distance in the open field [$F_{(1,16)} = 9.51$; $P < 0.01$], which was fully counterbalanced by a pretreatment with neopterin (0.4 pmol; i.c.v.) [$F_{(1,16)} = 5.28$; $P < 0.05$]. Fig. 3C shows that the effect of neopterin on aversive learning is also preserved in IL-$10^{-/-}$ mice, in both the STM and the LTM test sessions. In addition, the cytokine-induced motor impairment (Fig. 3D, E) also elicited a significant increase in the latency to step-down in the LTM session (Fig. 3C), in agreement with the reduced locomotion observed in LPS-treated mice (Fig. 3B). However, the neopterin-facilitated learning process seems the responsible for increased latency observed in LPS/neopterin treated mice, since spontaneous locomotion was fully preserved (Fig. 3B). Furthermore, the protective effects of neopterin on locomotor and memory impairments induced by LPS were also related to a significant reduced production of brain IL-6 [$F_{(1,3)} = 46.664$; $P < 0.001$], while IL-1β was not modified under these conditions by neopterin (Fig. 3D and E, respectively). This effect was also confirmed in wild type Swiss mice (Fig. 3F) [$F_{(3,21)} = 7.936$; $P < 0.001$]. The LPS treatment protocol used here is known to provoke increased cytokine gene expression in the brain, four hours after LPS stimulation (Suppl. Fig. 1).

3.4. Neopterin does not affect the consolidation of fear memories in C57BL/6 mice

Fig. 4A–E shows the effect of a single i.c.v. administration of neopterin (0.4 pmol) in C57BL/6 mice, following the experimental design shown in Fig. 4A. Fig. 4B–E shows that neopterin did not

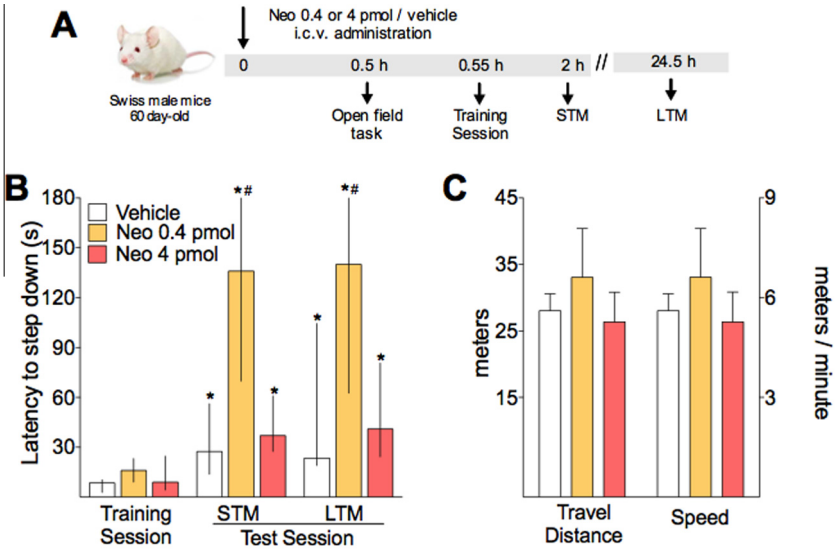

Fig. 1. Effect of neopterin on aversive memory in Swiss mice. A single neopterin dose (0.4 and/or 4 pmol) was intracerebroventricularly (i.c.v.) injected in Swiss mice. (A) Experimental design. (B) Latency to step down in the step-down test. $^{*}P < 0.05$, *vs.* acquisition session; $^{\#}P < 0.05$, *vs.* vehicle (Kruskal-Wallis followed by Dunn as *post hoc* test). (C) Travelled distance and velocity in the open field. Neo: neopterin; STM: short term memory; LTM: long term memory, (n = 10 animals per group).

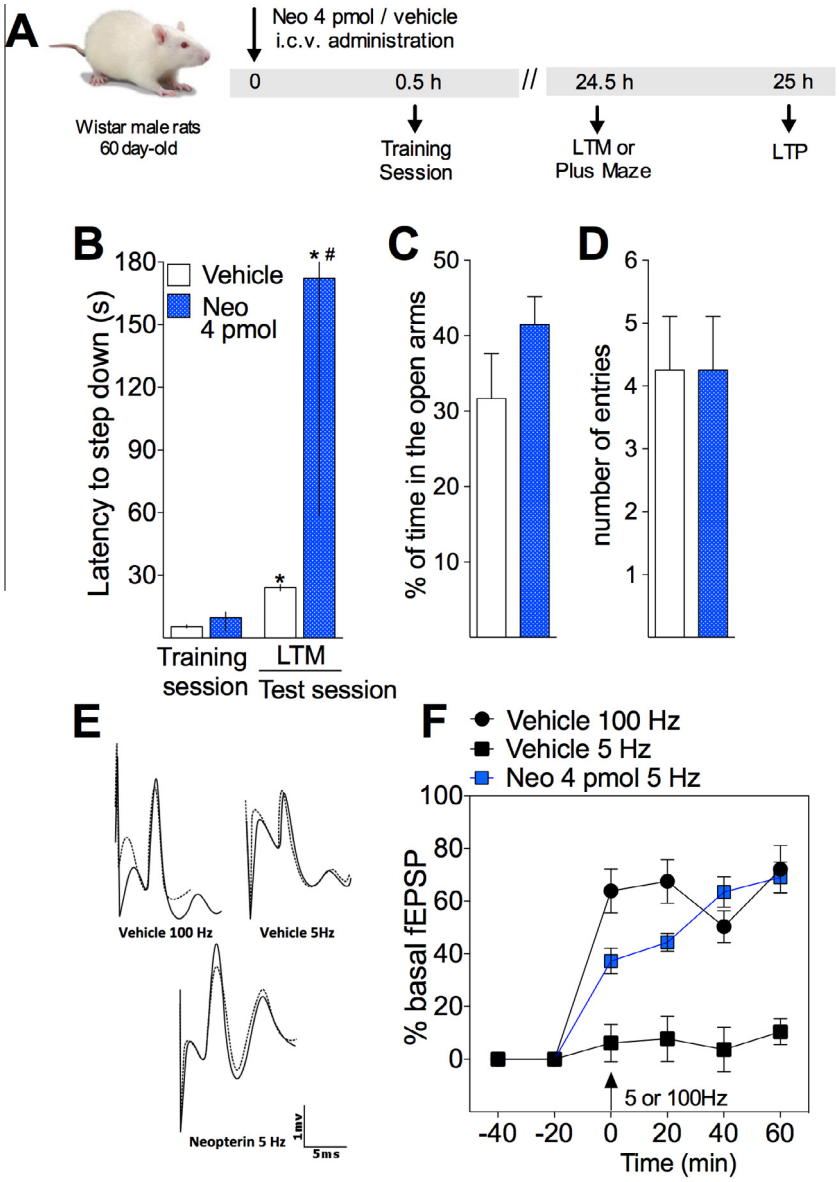

Fig. 2. Effect of neopterin on aversive memory in Wistar rats. A single neopterin dose (4 pmol) was intracerebroventricularly (i.c.v.) injected in Wistar rats. (A) Experimental design. (B) Latency to step down in the step-down test. $^{*}P < 0.05$ (Mann–Whitney *U* test), (n = 3 animals per group in vehicle and n = 6 animals per group in neopterin). (C) Percentage of time spent in open arms, and (D) number of entries in the closed arms of elevated plus maze apparatus (n = 4 animals per group). (E) *fEPSP* sample traces for vehicle and neopterin groups before (full line) and after (dotted line) effective tetanus. (F) Long-term potentiation formation (n = 4 animals per group). The time course graph shows the increments in field excitatory post-synaptic potentials (fEPSP), as % of basal fEPSP, after different frequency stimulation (5 or 100 Hz) protocols. Black arrow at time 0 min indicates when stimulation protocol was delivered. Neo: neopterin; STM: short term memory; LTM: long term memory.

alter the consolidation of new fear memories when injected after the acquisition phase (conditioning). It can be observed in the Fig. 4 that mice presented progressive enhanced memory retention, as indicated by a larger freezing time along the session (Fig. 4B and C). However, neopterin administration did not modify the freezing of animals at the contextual fear conditioning (context extinction test; Fig. 4D), or in the cued fear conditioning (tone test; Fig. 4E).

4. Discussion

The present data shows that a single i.c.v. injection of neopterin enhances learning and memory in different species of rodents under normal and inflammatory conditions. To our knowledge, this is the first evidence showing that an endogenous pteridine, currently considered to be an inert byproduct of inflammation, enhances cognition.

The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) defines cognitive deficiencies as a category of mental health disorders that primarily affect learning, memory, perception, and problem solving, and which includes amnesia, mild cognitive impairment, dementia, and delirium (Sachdev et al., 2014). Activation of the innate immune system and the production of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and INF-γ, have been recognized as important contributors to cognitive impairment (Reichenberg et al., 2001). Some common causes are Alzheimer's disease, cerebrovascular disease, Lewy body disease, frontotemporal degeneration, traumatic brain injury, infections, and alcohol abuse (Sachdev et al., 2014). Considering that neopterin is a sensitive biomarker of inflammation and increased levels of the compound have been reported in the

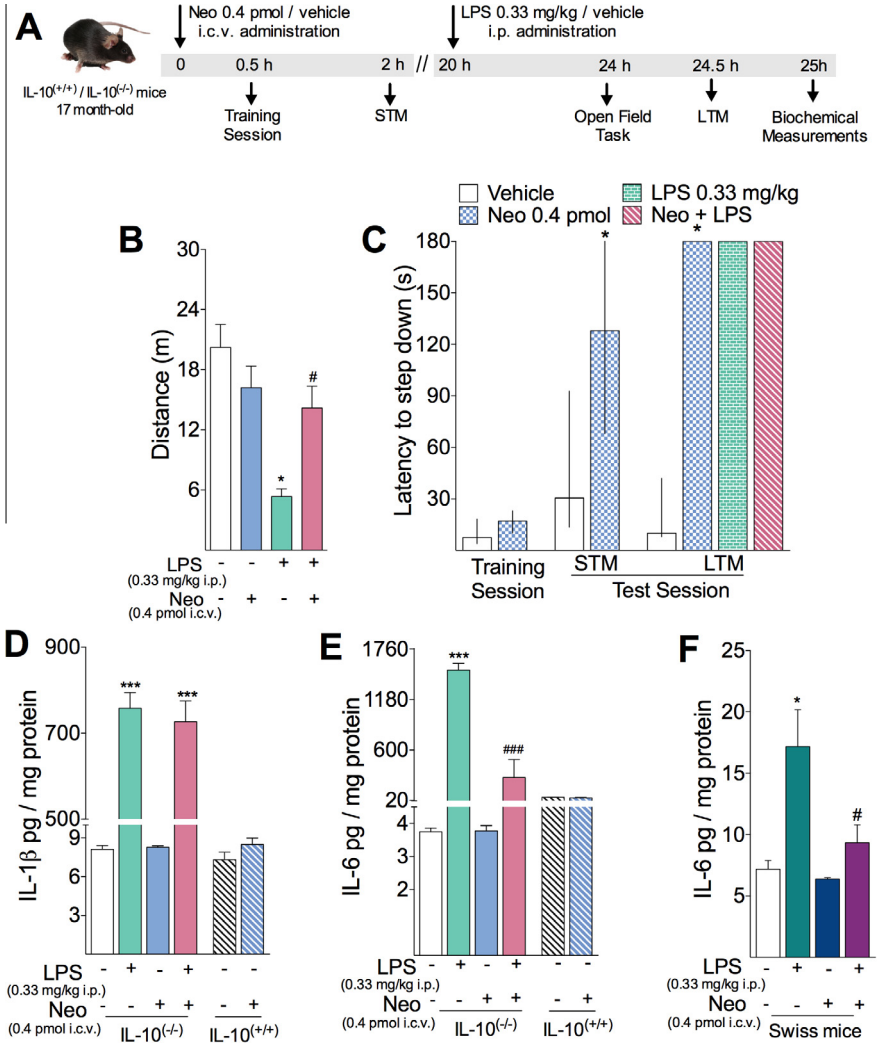

Fig. 3. Effect of neopterin on aversive memory in IL-10 null mice. A single neopterin dose (0.4 pmol) was intracerebroventricularly (i.c.v.) injected in IL-10 null mice previously to LPS injection (i.p.) (A) Experimental design. (B) Travelled distance in the open field test. (C) Latency to step down in the step-down test. (D) IL-1β, and (E) IL-6 levels in brain homogenates. (F) IL-6 levels in brain homogenates from male adult Swiss mice. $^{*}P < 0.05$, *vs.* vehicle; $^{\#}P < 0.05$, *vs.* LPS; $^{***}P < 0.001$, *vs.* vehicle; $^{\#\#\#}P < 0.001$, *vs.* LPS (Two-way ANOVA followed by Tukey as *post hoc* test). Latency data is represented as median with interquartile range, while fEPSP percentages and travelled distances as mean ± S.E.M. LPS: *Escherichia coli* lipopolysaccharide; Neo: neopterin; STM: short term memory; LTM: long term memory; (n = 3 or 4 animals per group).

cerebrospinal fluid of individuals affected by these conditions (Bechter et al., 2010; Denz et al., 1990; Firoz et al., 2015; Hagberg et al., 2010; Parker et al., 2013; Sucher et al., 2010), in the present study we explored whether the pteridine could induce changes in cognition, following central administration.

We found that neopterin treatment significantly improved hippocampal learning and memory in different rodent species. A well-established protocol to assess these processes in rodents is the one-trial step-down test, where animals learn to suppress the exploratory tendency to avoid aversive stimuli (Izquierdo and Medina, 1997). Moreover, the training session in the step-down test induces LTP generation in the hippocampus (Izquierdo and Medina, 1997; Walz et al., 1999, 2000), and it has been demonstrated to be essential for STM and LTM formations. LTP represents the acquisition and maintenance of memories at a synaptic level (Bliss and Collingridge, 1993; Izquierdo and Medina, 1997; Walz et al., 1999, 2000). Our observations demonstrated that neopterin, when administered prior to the training session (before acquisition, but not before consolidation), increased the latency to step-down, both in mice and rats, as a result of facilitated learning in the inhibitory avoidance task. The reduced threshold to generate LTP, following i.c.v. neopterin injection, further supports this response, indicating that this pteridine enhances hippocampal synaptic plasticity and is unlikely to involve locomotive impairments or anxiogenic effects. However, the effect of neopterin appears to be limited to certain forms of memory, since the administration of the pteridine after consolidation did not facilitate the learning process in the fear conditioning task.

Mechanistically, improved cognition mediated by neopterin might be related to the antioxidant (Ghisoni and Latini, 2015; Ghisoni et al., 2015) or anti-inflammatory properties of the molecule. The neopterin-elicited reduction of IL-6 production after an LPS-induced sickness behavior in IL-10 null mice, preserved spontaneous locomotion and also enhanced memory. In agreement, IL-6 null mice were resistant to behavioral deficits caused by tissue abscesses, viral infections (Kozak et al., 1997), or an LPS-challenge (Sparkman et al., 2006). On the other hand, it has been reported that increased peripheral levels of IL-6 were associated with poorer cognitive function and steeper cognitive decline in middle aged and elderly individuals (Economos et al., 2013; Marsland et al., 2006; Mooijaart et al., 2013; Reichenberg et al., 2001; Singh-Manoux et al., 2014). Thus, these findings strongly

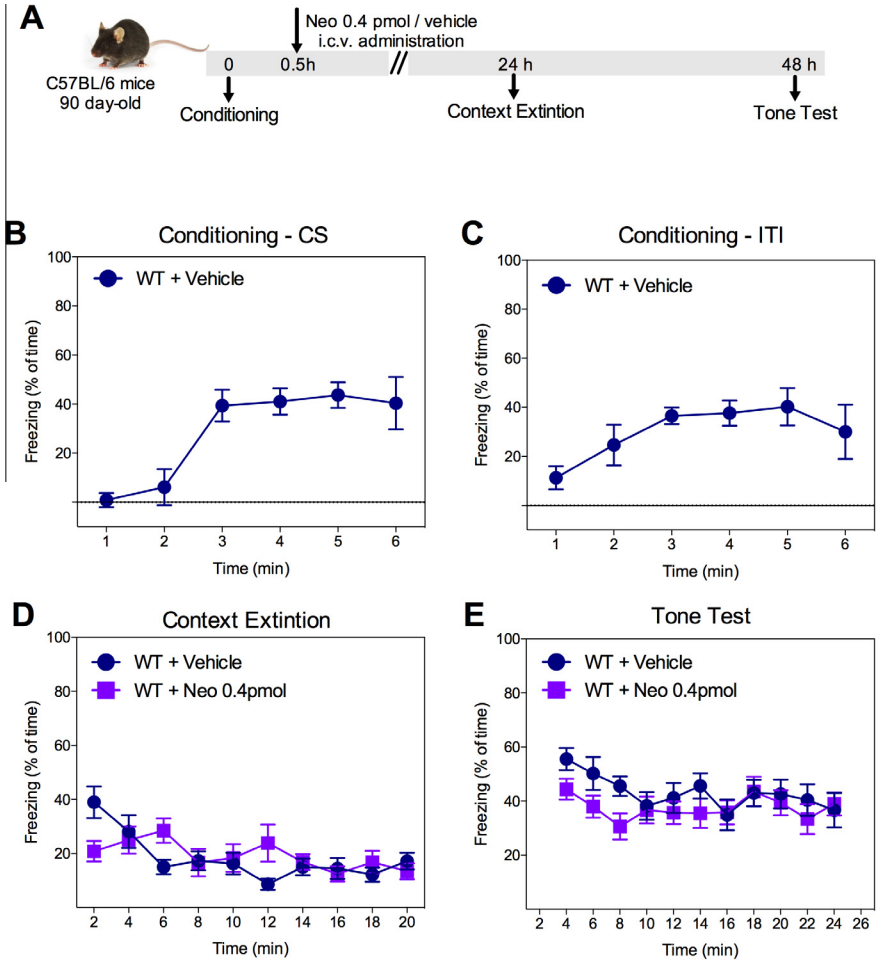

Fig. 4. Effect of neopterin on fear memory in C57BL/6 mice. (A) A single i.c.v. neopterin dose (0.4 pmol) was injected in C57BL/6 mice and fear conditioning was measured. For details see M&M. (B) Percentage of freezing during the conditioned stimulus (CS). (C) Percentage of freezing during the inter-trial interval (ITI). (D) Percentage of freezing during the context extinction. (E) Percentage of freezing during the tone test. Data are presented as mean ± S.E.M; (n = 12 animals per group).

suggest that reduced IL-6 production might also be related to neopterin's positive effects on learning and memory.

Alternatively, neopterin might facilitate learning and cognitive processes by activating the down-stream effects triggered by Nrf2, which would be in agreement with our previous observations of neopterin-induced HO-1 content in brain cells (Ghisoni et al., 2015). The involvement of this protective pathway in learning and memory has already been demonstrated in both animals and humans. For example, the use of the naturally-occurring activators of the Nrf-2 pathway, namely resveratrol and flavanone, improved learning and memory in rodents with vascular dementia (Liu et al., 2015b; Ma et al., 2013), red ginseng-enriched diets ameliorated age-related decline in mice models of learning and memory (Lee and Oh, 2015), and the administration of curcumin resulted in increased HO-1 content, associated also with enhanced learning in rats (Ataie et al., 2010; Socci et al., 1995; Xie et al., 2014). This promnesic effect has also been observed in adult humans (Cox et al., 2015; Witte et al., 2014). Therefore, further studies addressing the role of the Nrf2 pathway in neopterin's effects on learning and memory are certainly of great interest.

In the last decades, pharmacological treatments aimed at improving cognitive function have been explored and have in some cases become established in clinical practice (Parton et al., 2005). Some compounds have already been approved by the US Food and Drug Administration (FDA), including for example, memantine and galantamine (Mehlman, 2004), an uncompetitive N-methyl-D-aspartate receptor antagonist (Ma et al., 2015), and a cholinesterase inhibitor (Deardorff et al., 2015), respectively. Memory facilitators including natural compounds like vitamin E (An and Zhang, 2014; Khodamoradi et al., 2015), curcumin (Banji et al., 2014; Xie et al., 2014), omega-3 polyunsaturated fatty acids (Cutuli et al., 2014), or synthetic drugs such as galantamine (Ali et al., 2015) have also been linked to Nrf2 activation.

Our group has recently reported that neopterin *per se*, or in combination with a mitochondrial toxin, increased HO-1 content in striatal primary astrocytes. This suggests that neopterin possesses cytoprotective properties (Ghisoni et al., 2015), which is in agreement with the intrinsic relationship demonstrated by McNeill et al. (2015). The GTPCH deficient mice that the authors generated showed specific alterations in a set of 78 genes following LPS-nitric oxide synthase type 2 induction (NOS2), dependent on BH4 levels. In particular, Nrf2-dependent genes showed reduced induction in null mouse macrophages (McNeill et al., 2015). This demonstrates that BH4 is required to regulate the cellular redox state, a critical pathway for neopterin formation.

Therefore, it may be hypothesized that neopterin facilitates learning and memory, improving cognition by activating an antioxidant and anti-inflammatory environment. Finally, it is also feasible to propose that glial cells release neopterin in order to protect neurons during cellular stress. Microglial cells and

astrocytes are candidates to produce neopterin since these cells respond in a similar fashion as peripheral macrophages to inflammatory stimuli.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2016.02.019.

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