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Molecular mechanisms involved in interleukin 1-beta (IL-1β)-induced memory impairment. Modulation by alpha-melanocyte-stimulating hormone (α-MSH)

P. Gonzalez [a], I. Machado [a], A. Vilcaes [b], C. Caruso [c], G.A. Roth [b], H. Schiöth [d], M. Lasaga [c], T. Scimonelli [a,*]

[a] IFEC-CONICET, Depto. Farmacología, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina
[b] CIQUIBIC-CONICET, Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina
[c] Instituto de Investigaciones Biomédicas INBIOMED UBA-CONICET, Facultad de Medicina, Buenos Aires, Argentina
[d] Department of Neuroscience, Uppsala University, Uppsala, Sweden

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ABSTRACT

Pro-inflammatory cytokines can affect cognitive processes such as learning and memory. Particularly, interleukin-1β (IL-1β) influences the consolidation of hippocampus-dependent memories. We previously reported that administration of IL-1β in dorsal hippocampus impaired contextual fear memory consolidation. Different mechanisms have been implicated in the action of IL-1β on long-term potentiation (LTP), but the processes by which this inhibition occurs in vivo remain to be elucidated. We herein report that intrahippocampal injection of IL-1β induced a significant increase in p38 phosphorylation after contextual fear conditioning. Also, treatment with SB203580, an inhibitor of p38, reversed impairment induced by IL-1β on conditioned fear behavior, indicating that this MAPK would be involved in the effect of the cytokine. We also showed that IL-1β administration produced a decrease in glutamate release from dorsal hippocampus synaptosomes and that treatment with SB203580 partially reversed this effect. Our results indicated that IL-1β-induced impairment in memory consolidation could be mediated by a decrease in glutamate release. This hypothesis is sustained by the fact that treatment with D-cycloserine (DCS), a partial agonist of the NMDA receptor, reversed the effect of IL-1β on contextual fear memory. Furthermore, we demonstrated that IL-1β produced a temporal delay in ERK phosphorylation and that DCS administration reversed this effect. We also observed that intrahippocampal injection of IL-1β decreased BDNF expression after contextual fear conditioning. We previously demonstrated that α-MSH reversed the detrimental effect of IL-1β on memory consolidation. The present results demonstrate that α-MSH administration did not modify the decrease in glutamate release induced by IL-1β. However, intrahippocampal injection of α-MSH prevented the effect on ERK phosphorylation and BDNF expression induced by IL-1β after contextual fear conditioning. Therefore, in the present study we determine possible molecular mechanisms involved in the impairment induced by IL-1β on fear memory consolidation. We also established how this effect could be modulated by α-MSH.

1. Introduction

Bidirectional communication between the central nervous system (CNS) and the immune system has been extensively studied, and this has attracted interest in the role that certain cytokines, particularly the proinflammatory cytokine interleukin-1β (IL-1β), might play in the brain. The finding that IL-1 type I receptor (IL-1RI) is expressed in high density in the hippocampus (French et al., 1999), an area of the brain that plays an important role in memory and learning (Eichenbaum et al., 1996), has prompted studies on the role of IL-1β in cognitive function. There is growing evidence demonstrating that high levels of IL-1β in the brain could impair memory consolidation (Rachal Pugh et al., 2001; Yirmiya and Goshen, 2011), as well as cytokine participation in some detrimental changes in cognitive functions during neurodegenerative diseases (Holmes et al., 2003; Nagatsu and Sawada, 2005). In contrast, physiological levels of IL-1β promote long-term potentiation (LTP) and memory formation (Avital et al., 2003; Yirmiya et al., 2002). All together, these findings suggest that the influence of IL-1β on memory follows an inverted U-shaped pattern; basal

* Corresponding author. Address: IFEC-CONICET, Departamento de Farmacología, Facultad de Ciencias Químicas, Haya de la Torre y Medina Allende Ciudad Universitaria, Universidad Nacional de Córdoba, 5000 Córdoba, Argentina. Tel.: +54 351 5353852x3154; fax: +54 351 4334420.
E-mail address: tscimonelli@mail.fcq.unc.edu.ar (T. Scimonelli).

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IL-1β levels are needed for memory consolidation. However, any deviation, either due to an increase in IL-1β levels (induced by immune, physical, or psychological challenge) or blockade of IL-1β signaling, results in memory impairment (Goshen and Yirmiya, 2007).

IL-1RI is expressed on glia (astrocytes and microglia), particularly after injury (Friedman, 2001), and also on specific populations of neurons, including hippocampal pyramidal neurons (Ericsson et al., 1995). IL-1β elicits distinct functional responses in these different CNS cell types. It has been suggested that IL-1β regulates the production of inflammatory cytokines in glia while also influencing synaptic function in hippocampal neurons (Bellinger et al., 1993; Srinivasan et al., 2004). In astrocytes, IL-1β induced both p38 mitogen-activated protein kinases (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) pathways regulating inflammatory response; however, in hippocampal neurons IL-1β activated p38 but not NF-κB (Srinivasan et al., 2004). Recently, a novel isoform of interleukin-1 receptor accessory protein (IL-1RAcP), IL-1RAcPb, was found exclusively in CNS neurons. IL-1RAcPb was shown to play a modulatory role in the activation of p38 MAPK but had no effect on NF-κB signaling (Smith et al., 2009).

Many mechanisms have been implicated in the inhibitory effect of IL-1β on LTP; however, the mechanisms by which this inhibition occurs during memory consolidation are not yet clearly elucidated. IL-1β was shown to inhibit glutamate release (Kelly et al., 2003; Vereker et al., 2000) and reduce calcium influx in hippocampal synaptosomes (Plata-Salaman and Ffrench-Mullen, 1992). Also, the inhibition of LTP induced by IL-1β was coupled with stimulation of the p38 MAPK in hippocampus and entorhinal cortex, an effect abrogated by the p38 inhibitor SB203580 (Kelly et al., 2003).

Brain-derived neurotrophic factor (BDNF) is capable of affecting memory processes due to its critical role in synaptic plasticity processes (Barco et al., 2005; Patterson et al., 1996) and long term memory (Hall et al., 2000; Mizuno et al., 2000). Furthermore, BDNF is rapidly and selectively induced in hippocampus following contextual fear conditioning (Hall et al., 2000), and systemic administration of either IL-1β or lipopolysaccharide (LPS) or stress-induced elevations of IL-1β in hippocampus (Nguyen et al., 1998; Pugh et al., 1999) decreases BDNF mRNA in this area (Barrientos et al., 2003; Lapchak et al., 1993). Moreover, intra-hippocampal administration of the IL-1 receptor antagonist (IL-1ra), prevents both BDNF downregulation and memory impairment (Barrientos et al., 2003).

A variety of effects of central IL-1β administration are blocked by α-melanocyte stimulating hormone (α-MSH) (Cragnolini et al., 2004, 2006). α-MSH and other melanocortins (β-MSH, γ-MSH and ACTH) are members of a family of endogenous peptides derived from pro-opiomelanocortin (POMC). The effects of melanocortins are mediated by five different receptors (MC1, MC2, MC3, MC4, MC5) that belong to the G protein coupled receptor family and are functionally coupled to adenylate cyclase (Catania et al., 2010; Holder and Haskell-Luevano, 2004). The effects of their activation are mediated primarily by cAMP-dependent signaling pathway. The stimulated cAMP production activates protein kinase A and cAMP response element-binding (CREB) phosphorylation (Caruso et al., 2012; Sarkar et al., 2002). However, melanocortin signaling is also conveyed through cAMP-independent pathways (Chai et al., 2006; Newman et al., 2006; Vongs et al., 2004). The MC4 receptor (MC4R) is expressed in virtually all CNS regions including the hippocampus (Kishi et al., 2003). MC4R mediate the antipyretic effect of melanocortins and their anti-inflammatory actions within the brain (Caruso et al., 2007; Sinha et al., 2004). Also, α-MSH through the activation of MC4R was able to reverse the effect of IL-1β on the consolidation (Gonzalez et al., 2009) and reconsolidation of a contextual fear memory (Machado et al., 2010).

Therefore, the objective of this study was to identify molecular mechanisms induced by intrahippocampal administration of IL-1β that might lead to impairment of memory consolidation. We analyzed p38 activation and the effect of IL-1β on mechanisms critically involved in memory consolidation such as glutamate release and ERK phosphorylation. We also evaluated the modulatory role of α-MSH on these effects.

2. Methods

2.1. Animals

Adult male Wistar rats weighing 270–300 g at the time of surgery were used for these studies. All animals were housed in standard laboratory plastic cages in groups of three per cage with food and water available *ad libitum*. Animals were kept on a 12 h light/dark cycle (lights on 7:00–19:00 h) with a constant room temperature of 22 ± 1 °C. Behavioral testing was performed during the light cycle between 10:00 and 14:00 h. Procedures were performed according to the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals as approved by the School of Chemical Sciences, National University of Cordoba Animal Care and Use Committee. The number of animals used as well as their suffering was kept to the minimum possible needed to accomplish the goals of this study.

2.2. Surgery and drug infusion procedures

Animals were anesthetized with an intraperitoneal (ip) injection of ketamine hydrocloride (55 mg/kg) and xylazine (11 mg/kg) and placed in a stereotaxic instrument (Stoelting, Wood Dale, IL) with the incisor bar set at −3.3 mm. Rats were implanted bilaterally with stainless-steel guide cannulas (22 gauge; length, 9 mm) in the dorsal CA1 region of the hippocampus on coordinates antero posterior (AP): −3.3 mm; lateral (L): ±2.0 mm; dorso ventral (DV): −2.5 mm according to the rat brain atlas (Paxinos and Watson, 2007). Cannulas were fixed to the skull with a screw and dental acrylic. Animals were removed from the stereotaxic instrument and recovered under a heat lamp and close supervision. After surgery, animals were gently handled every day and habituated to intrahippocampal injections throughout the recovery period for 7 days before experimental procedures.

To perform local infusions into the hippocampus, rats were hand-restrained, and drugs or vehicle were administered with infusion cannulas (30 gauge) fitted into and extending 1 mm beyond the guide cannulas. The infusion cannulas were connected, via polyethylene tubing (PE 10; Becton Dickinson, Sparks, MD), to 10 μl microsyringes (Hamilton, Reno, NV) mounted on a microinfusion pump (Stoelting, Illinois, USA). Each rat was bilaterally injected with 0.25 μl per side at a flow rate of 0.25 μl/min. To allow diffusion of the drug, infusion cannulas were kept in place for another minute.

2.3. Drugs

The drugs used in these experiments were rrIL-1β (rat recombinant IL-1β) (R&D Systems, USA), α-MSH (NeoMPS, France), SB203580 Hydrochloride (Calbiochem, USA) and D-cycloserine (DCS) (Sigma Aldrich, USA). The rrIL-1β was dissolved in sterile 0.9% saline containing 0.01% bovine serum albumin and stored in aliquots at −70 °C. α-MSH and SB203580 were dissolved in sterile 0.9% saline and stored in aliquots at −20 °C. DCS was dissolved in sterile saline for intraperitoneal (i.p. injection [1 ml/kg]) immediately before it was used. Rats received bilateral hippocampal infusions of the different drugs in the following doses: IL-1β (5 ng/0.25 μl), α-MSH (0.05 μg/0.25 μl), SB203580 (100 μM). DCS was administered in doses of 3, 7.5 or 15 mg/kg. Sterile saline (SAL)

was used for control injections and administered bilaterally in hippocampus (0.25 μl) or i.p. (1 ml/kg).

2.4. Histological procedures

At the end of behavioral tests, rats were killed with an overdose of 16% chloral hydrate, decapitated, and brains removed for immersion-fixation in a 4% formalin solution. Frontal sections were cut in a cryostat (Leica, Nussloch, Germany) for localization of injection sites, and extent of tissue damage was examined under a light microscope. Only animals with proper injection sites and tissue damage not exceeding the diameter of the cannulas were included in the study.

2.5. Contextual fear conditioning

Classical fear conditioning is a paradigm in which animals learn to fear an initially neutral stimulus, called conditioned stimulus (CS). The CS, such as a context, is presented with an aversive unconditional stimulus (US) such as a footshock. The pairing of the CS and the US comes to elicit conditioned fear responses including increased freezing behavior.

2.6. Apparatus

The conditioning chamber was made of white acrylic ($20 \times 23 \times 20$ cm) with transparent lid. The floor consisted of 10 parallel stainless steel grid bars, each measuring 4 mm in diameter and spaced 1.5 cm apart (center to center).The grid floor was attached to a scrambled shocker to provide footshock. Illumination was provided by a 2.5 W white light bulb. The chamber was placed in a room illuminated by a white fluorescent tube located on the ceiling and was cleaned with 30% ethanol in water before and after each use. Background noise was supplied by ventilation fans and shock scramblers. Experiments were always performed between 10:00 and 14:00 h with experimenters unaware of the treatment condition.

2.7. Procedure

Fear conditioning was performed on the 7th day after surgery. Training consisted in placing the rat individually in the conditioning chamber where it was left undisturbed for a 3 min acclimatization period (pre-shock period). After this period, rats received three unsignaled footshocks (0.4 mA; 2.5 s duration; 30 s inter-shock interval). Animals remained in the chamber for an additional 2 min (post-shock period) and immediately afterwards were placed in their home cages and returned to the colony room.

For the contextual fear test, animals were reintroduced into the conditioned context for a 5 min period without shock delivery, 24 h after training. Memory was assessed and expressed as the percentage of time rats spent freezing. This behavior, commonly used as an index of fear in rats (Blanchard and Blanchard, 1989), was observed during this exposure period. An animal was considered to be freezing when it was crouching without movement of the body or head, except that associated with breathing. The total time spent freezing in each period was quantified (in seconds) using a stopwatch and expressed as percentage of total time.

2.8. Western blotting

Animals were killed by decapitation at different time after conditioning according the experiment: 0, 15 or 30 min for p38 determination; 15, 30 and 120 min for ERK analysis and 180 min after conditioning for BDNF determination.

The brains quickly removed and placed in an acrylic brain matrix (Stoelting CO., USA) on ice. Coronal brain slices of 4 mm were made 2 mm before and 2 mm after the guide cannula trace. Then, the dorsal hippocampus was dissected using the rat brain atlas (Paxinos and Watson, 2007) as a guide and the total proteins were extracted as follows. The hippocampus tissue were homogenized in RIPA's buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulphate) containing protease inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 100 μg/ml phenylmethylsulfonyl fluoride) and phosphatase inhibitor (1 mM sodium orthovanadate) and centrifuged at 10,000*g* for 15 min at 4 °C. Supernatants were combined with an equal volume of Laemmli's buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol,125 mM Tris, pH 6.8, 0.01% bromophenol blue), boiled for 5 min at 100 °C and stored at −70 °C until use. Aliquots of each sample were used for total protein quantification according to Bradford assay (Bio-Rad Bradford Protein Assay Kit; Hercules, CA). Protein samples (35 μg/lane) were separated by 10% (for the MAPKs ERK or p38) or 15% (for BDNF) SDS–PAGE and transferred onto polyvinylidene fluoride membrane (BioRad).

For detection of phosphorylated ERK, phosphorylated p38 and BDNF, immunoblots were blocked in TTBS buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) with 5% powdered low-fat milk. Then, they were incubated overnight at 4 °C with a rabbit monoclonal anti-phospho-p44/42 MAPK (1:1000, Cell Signaling Technology); rabbit monoclonal anti-phospho-38 MAPK (1:500, Cell Signaling Technology) or rabbit polyclonal anti-BDNF (1:1000, Santa Cruz Biotechnology, Inc), respectively. Blots were then incubated with anti-rabbit conjugated to horseradish peroxidase (1:2500, Jackson) for 120 min at rt and developed using enhanced chemiluminescence on X-ray film. Immunoblots were then stripped with 100 mM β-mercaptoethanol and 2% SDS in 62.5 mM Tris–HCl, pH 6.8, for 30 min at 55 °C. For detection of total ERK, total p38 and total actin the stripped blots were re-blocked and probed overnight at 4 °C with a mouse monoclonal anti-p44/42 MAPK (1:2500, Cell Signaling Technology); rabbit polyclonal anti-p38 MAPK (1:1000, Cell Signaling Technology) or mouse monoclonal anti-actin (1:2000, Sigma–Aldrich, Inc.) respectively. Blots were then incubated with anti-mouse or anti-rabbit conjugated to horseradish peroxidase (1:2500, Jackson) for 120 min at rt. The resulting film samples were scanned and analyzed with an image analysis program (Gel-Pro Analyzer Software). Data are presented as ratio of phospho-ERK2/total ERK2; phospho-p38/total p38; BDNF/total actin in each sample. Although the anti-phospho and anti-total ERK1/2 antibodies used in the present study recognize both ERK1 and ERK2, p-ERK1 was not quantified because the p-ERK1 signals were often too faint and inconsistent to be accurately analyzed.

3. Glutamate release

3.1. Preparation of hippocampal synaptosomes

The animals were sacrificed by decapitation five min after the treatments. The dorsal hippocampus was dissected as previously described and synaptosomes were purified on discontinuous Percoll gradients as described previously (Dunkley et al., 2008). Synaptosomes sedimenting between 10% and 23%. Percoll bands were collected and diluted in a final volume of 10 ml of HEPES buffer medium consisting of 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 10 mM glucose and 10 mM HEPES, pH 7.4 before centrifugation at 22,000*g* for 14 min at 4 °C. The pellets were resuspended in 1 ml of HEPES buffer medium, and protein content was determined by Bradford assay (Bio-Rad

Bradford Protein Assay Kit; Hercules, CA). Synaptosomal pellets were stored on ice and used within 3–4 h.

3.2. Glutamate release assay

Glutamate release was monitored on-line with an assay using exogenous glutamate dehydrogenase and $NADP^+$ to couple the oxidative decarboxylation of glutamate released. Then, the NADPH generated was detected fluorometrically (Nicholls et al., 1987; Vilcaes et al., 2009). Briefly, synaptosomal pellets were resuspended in HEPES buffer medium and incubated in a stirred and thermostated cuvette maintained at 37 °C in FluoroMax-P Horiba Jobin Yvon Spectrofluorimeter (Horiba Ltd, Kyoto, Japan). Then, 1 mM $NADP^+$, 50 units/ml glutamate dehydrogenase and 1.2 mM $CaCl_2$ were added after 3 min. After 5 min of incubation, 3 mM 4AP was added to stimulate glutamate release. Traces were calibrated by the addition of 4 nmol of glutamate at the end of each assay. Data points were obtained at 1-s intervals.

3.3. Statistics

All data are expressed as mean ± standard error (S.E.M.) and were analyzed by one-way or two-way ANOVA as appropriate. If any statistically significant difference was found, post hoc analysis was performed using Newman Keuls or LSD Fisher multiple comparison test. Differences with p values lesser than 0.05 were considered statistically significant.

4. Results

4.1. p38 MAPK activation is involved in the impairment induced by IL-1β on contextual fear conditioning

To assess whether p38 signaling pathway mediated IL-1β effect on memory consolidation we determined phosphorylation of this MAPK in the dorsal hippocampus. Results showed that IL-1β (5 ng/0.25 μl) induced phosphorylation of p38 MAPK 15 min after treatment, which declined thereafter. Furthermore no modification in phospho-p38MAPK levels was found at any time studied (0, 15 or 30 min) in the hippocampus of saline injected animals (Fig 1). No change in total p38 MAPK could be detected at any post-training time studied. Two way ANOVA indicated a significant effect for interaction between treatment and time [$F(2,25) = 5.64$; $p < 0.01$]. The post hoc test Newman Keuls revealed that there were no significant changes in p38 phosphorylation in control animals until 30 min post conditioning and that IL-1β administration produced a significant increase in p38 activation 15 min after conditioning, compared to time 0 min.

As previously reported, administration of IL-1β (5 ng/0.25 μl) in dorsal hippocampus caused a reduction of freezing response, an effect attenuated by pre-treatment with p38 inhibitor SB203580 (100 μM). Injection of SB203580 (100 μM) alone had no significant effect on the freezing percentage measured during the test (Fig. 2). One way ANOVA showed a significant effect for the treatment [$F(3,39) = 3.53$; $p = 0.024$]. The post hoc Neuman Keuls test indicated that injection of IL-1β produce a decrease in percent of freezing and that there were no significant differences between groups treated with saline-saline, SB203580- saline or SB203580-IL-1β.

4.2. IL-1β reduced glutamate release from hippocampal synaptosomes after contextual fear conditioning

We determined glutamate release in synaptosomes prepared from dorsal hippocampus of rats treated with saline or IL-1β (5 ng/0.25 μl) immediately after conditioning. Two additional

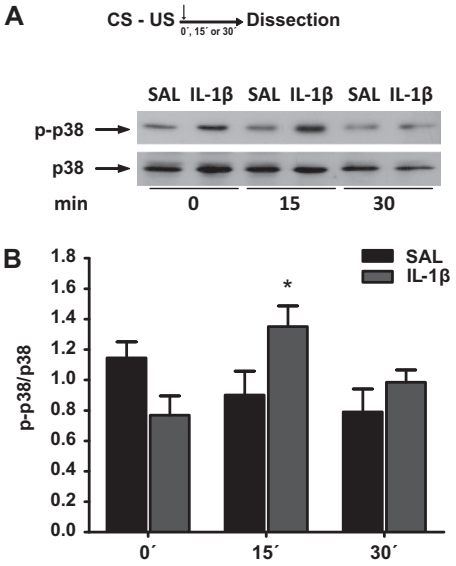

Fig. 1. Effect of IL-1β on p38 MAPK phosphorylation after contextual fear conditioning. Animals were injected immediately after contextual fear conditioning with saline or IL-1β (5 ng) and killed 0, 15 or 30 min after training. MAPK p38 phosphorylation was assessed by densitometric analysis of western blot in total homogenates prepared from dorsal hippocampus. (A). Representative western blot of p-p38 and total p38 levels. (B). Data are expressed as mean ± S.E.M. of p-p38/p38 ratio. $n = 5$–6 per group. $^*p < 0.05$ versus. IL-1 T0 group.

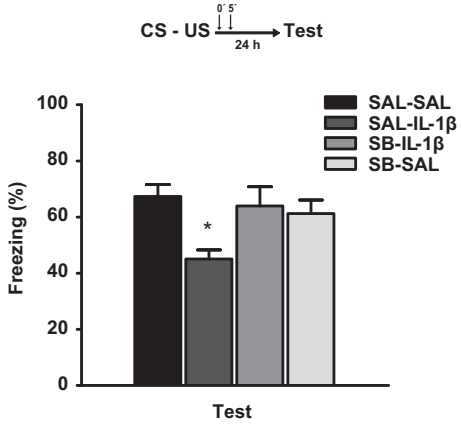

Fig. 2. Effect of SB203580, an inhibitor of p38 phosphorylation, on impairment induced by IL-1β on contextual fear conditioning. Rats were treated in dorsal hippocampus immediately after contextual fear conditioning with saline or SB203580 (100 μM) and 5 min later received another injection of saline or IL-1β (5 ng). Data are represented as mean ± S.E.M percentage of time rats spent freezing 24 h after conditioning during the test. $n = 9$–12 per group. $^*p < 0.05$ versus other groups.

groups were included: naive (rats killed without subjection to any behavioral protocol) and shock (animals that were placed in the conditioning cage, received a shock and were immediately removed from the cage). Both groups were treated with saline.

Contextual fear conditioning produced an increase in glutamate release from dorsal hippocampal synaptosomes (Fig 3). Glutamate release from synaptosomes prepared from naive and shock groups was significantly lower than glutamate release from synaptosomes from animals trained in fear conditioning. Our results corroborated that glutamate released increased after fear conditioning, and that this effect was related to the association between context and shock. Treatment with IL-1β reduced glutamate release compared to control group (Fig 3). Analysis of endogenous glutamate release

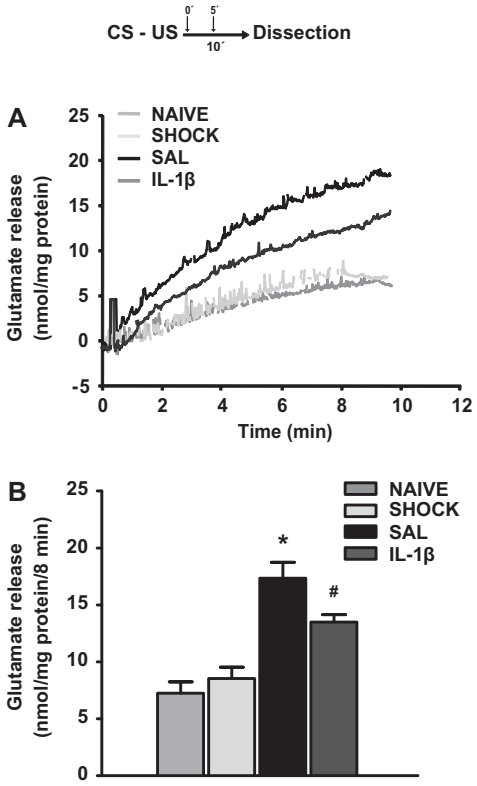

Fig. 3. Effect of IL-1β on glutamate release induced by 4-AP from hippocampal synaptosomes after contextual fear conditioning. Animals were treated in dorsal hippocampus immediately after contextual fear conditioning with saline or IL-1β (5 ng). Two additional control groups were injected with saline: naive group (rats not subjected to any behavioral protocol) and shock group (rats that only received a shock and were immediately removed from the cage). All groups received 5 min later an injection of saline. Five min after injections rats were killed and glutamate release was assessed in synaptosomes prepared from dorsal hippocampus. (A) Traces are representative of independent experiments. (B) Values registered after 8 min were expressed as mean ± SEM of 3–6 independent experiments. $^{*}p < 0.05$ versus. other groups. $^{\#}p < 0.05$ versus. other groups.

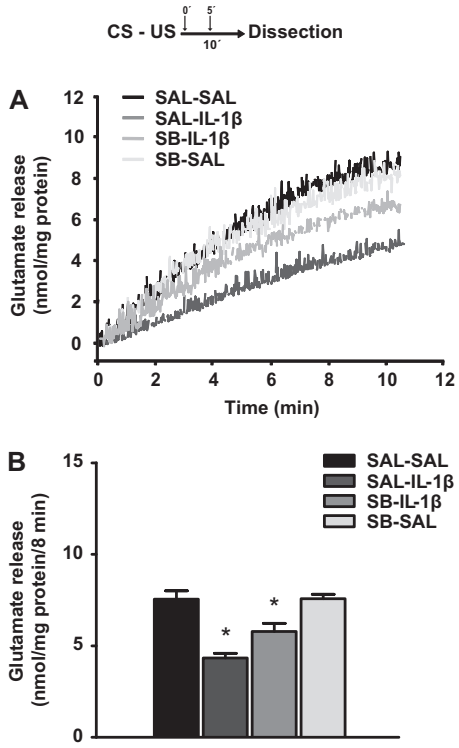

Fig. 4. Effect of SB203580 on glutamate release reduction induced by IL-1β after contextual fear conditioning. Immediately after contextual fear conditioning rats were treated with saline or SB203580 (100 μM) and 5 min later with saline or IL-1β (5 ng). Animals were killed 5 min after injections and the glutamate release induced by 4-AP was assessed in synaptosomes prepared from dorsal hippocampus. (A) Traces represent independent experiments. (B) Registered data after 8 min were expressed as mean ± SEM of 4–6 independent experiments. $^{*}p < 0.05$ versus. other groups. $^{\#}p < 0.05$ versus. other groups.

by one way ANOVA revealed a significant effect of treatment [$F(3,15) = 20.45$; $p < 0.001$]. The post hoc analysis by Newman Keuls test indicated that glutamate release was similar in naive and shock groups and significantly different from saline and IL-1β groups. There was also a significant decrease in glutamate release in animals that were conditioned and received IL-1β with respect to the control group that receive only saline.

4.3. Treatment with SB203580 partially reversed the effect of IL-1β on glutamate release

To determined whether changes observed in glutamate release and p38 activity were in fact associated, we measured glutamate release from synaptosomes of animals conditioned and immediately treated with SB203580 (100 μM) and 5 min later with IL-1β (5 ng/0.25 μl) in dorsal hippocampus.

IL-1β-induced decrease of glutamate release was partially attenuated by p38 inhibitor SB203580 (Fig 4). Treatment with SB203580 (100 μM) alone did not modify glutamate release. One way ANOVA indicated a significant effect of the treatment [$F(3,16) = 12.58$; $p < 0.001$]. The post hoc Newman Keuls test showed that glutamate release after treatment with IL-1β was lower than both control and SB203580 groups. Glutamate release from synaptosomes of animals treated with SB203580 and IL-1β was also statistically different from control and IL-1β groups.

4.4. IL-1β-induced memory impairment could be mediated by a decrease in glutamate release

The results described suggest that IL-1β-induced memory impairment could be mediated by a decrease in glutamate release. In order to confirm this hypothesis, we administered D-cycloserine, (DCS) a partial agonist of NMDA receptor. Different groups of animals were injected after conditioning with different doses of DCS (3 mg/kg; 7.5 mg/kg or 15 mg/kg ip). The dose of 15 mg/kg induced an increase in the percentage of time rats spent freezing 24 h after conditioning. Lower doses produced no changes on freezing during the test (Fig. 5A). One way ANOVA indicated a significant effect of the treatment [$F(3,31) = 4.24$; $p = 0.013$]. Newman Keuls test showed that DCS 15 mg/kg increased percent of freezing and that there was no significant difference between the other groups (control and DCS 3 or 7.5 mg/kg).

Considering these results, other groups of animals received 7.5 mg/kg of DCS i.p and 15 min later intra-hippocampal injections of saline or IL-1β (5 ng/0.25 μl). Treatment with DCS, at a dose that did not induce changes in freezing per se, reversed the effect of IL-1β on contextual fear memory, supporting that IL-1β-induced memory impairment could be mediated by a decrease in glutamate release (Fig. 5B). Two-way ANOVA indicated a significant effect of the second treatment [$F(1,32) = 6,53$; $p = 0.015$], and for the interaction between treatments [$F(1,32) = 5,62$; $p = 0.024$]. Newman Keuls test showed that IL-1β induced a decrease in percent of freezing, but behavior was similar in the others groups (DCS 7.5-IL-1β, IL-1β-saline and saline-saline).

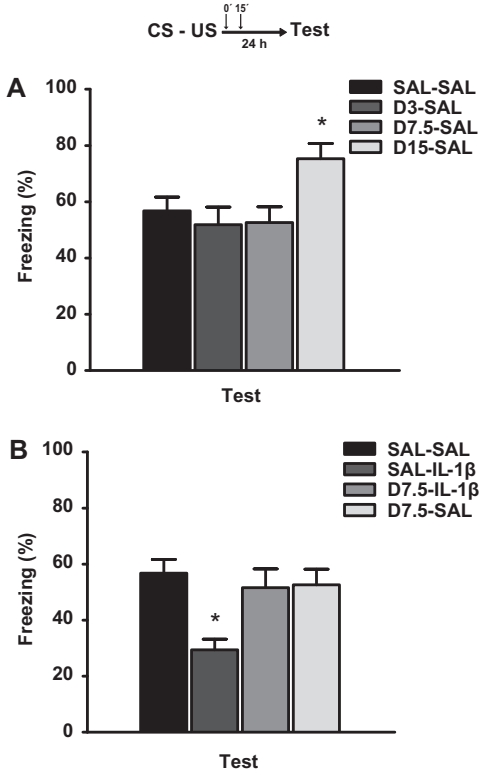

Fig. 5. Effect of D-cycloserine on impairment induced by IL-1β on contextual fear conditioning. (A) Different groups of animals were injected i.p. after conditioning with different doses of DCS (3; 7.5 or 15 mg/kg) and 15 min later received intra-hippocampal injections of saline. (B) Other groups were treated i.p. with saline or DCS 7.5 mg/kg and 15 min after in dorsal hippocampus with saline or IL-1β (5 ng). Data are the mean ± S.E.M percentage of time rats spent freezing 24 h after conditioning during the test. $n = 7–10$ per group. $^{*}p < 0.05$ versus other groups.

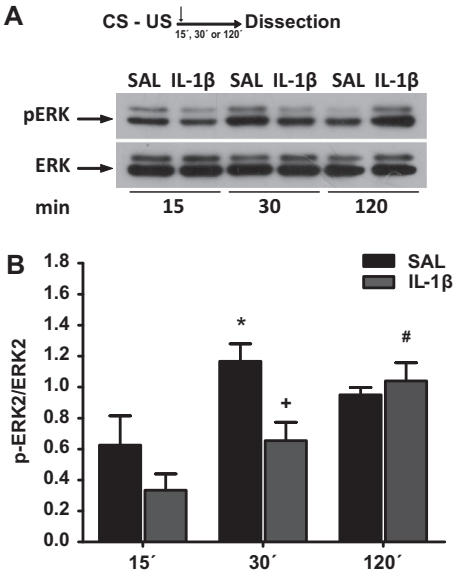

Fig. 6. Effect of IL-1β on ERK MAPK phosphorylation after contextual fear conditioning. Rats were injected immediately after contextual fear conditioning with saline or IL-1β (5 ng) and killed 15, 30 or 120 min after training. ERK2 phosphorylation was analyzed by western blot in total homogenates prepared from dorsal hippocampus. (A) Representative western blot of p-ERK2 and total ERK2 levels. (B) Data are expressed as mean ± S.E.M. of p-ERK2/ERK2 ratio. $n = 5–7$ per group. $^{*}p < 0.05$ versus. saline T15. $^{\#}p < 0.05$ compared to IL-1β groups. $^{+}p < 0.05$ versus saline T30.

4.5. IL-1β produces a temporal delay in ERK phosphorylation after contextual fear conditioning

We next examined ERK phosphorylation in dorsal hippocampus after contextual fear conditioning. Western immunoblotting analysis was performed in different groups of rats that were trained, received different treatments (saline or IL-1β (5 ng/0.25 μl) and were killed at different times (15, 30 and 120 min). after contextual fear conditioning.

There was a significant increase in ERK phosphorylation in control rats 30 min after conditioning, and treatment with IL-1β inhibited this increase. However, we observed an increase in ERK activation 120 min after training in IL-1β-treated rats (Fig 6). Two-way ANOVA indicated a significant effect of treatment [$F(1,30) = 5{,}90$; $p = 0.02$], time [$F(2,30) = 9.72$; $p < 0.001$] as well as interaction between treatment and time [$F(2,30) = 3.50$; $p = 0.04$]. The post hoc Fisher's LSD test demonstrated a significant difference in ERK phosphorylation between IL-1β treated animals and control group at 30 min. When pERK was analyzed 120 min after conditioning, levels were similar in both groups. Expression of total ERK did not differ between the different groups (Fig. 6).

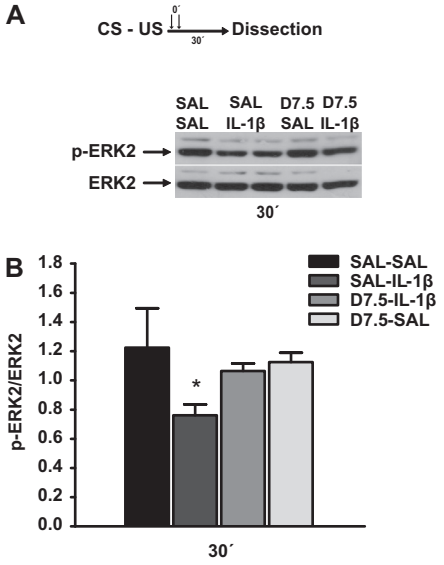

Fig. 7. Effect of D-cycloserine on ERK phosphorylation reduction induced by IL-1β after contextual fear conditioning. Different groups of animals were injected i.p. with saline or DCS (7.5 mg/kg) immediately after contextual fear conditioning and 1 min later received intra-hippocampal injections of saline or IL-1β (5 ng). Rats were killed 30 min after conditioning and ERK2 phosphorylation was analyzed by western blot in total homogenates prepared from dorsal hippocampus. (A) Representative western blot of p-ERK2 and total ERK2 levels. (B) Data are expressed as mean ± S.E.M. of p-ERK2/ERK2 ratio. $n = 3–5$ per group. $^{*}p < 0.05$ versus other groups.

4.6. Decrease in ERK2 phosphorylation induced by IL-1β after contextual fear conditioning is reversed by DCS

The animals were injected i.p. with saline or DCS (7.5 mg/kg) immediately after contextual fear conditioning and 1 min later they received intra-hippocampal injections of saline or IL-1β. Rats were killed 30 min after conditioning. Concordant with our previous results, DCS treatment reversed the decrease in ERK phosphorylation induced by IL-1β 30 min after conditioning (Fig 7). One way ANOVA determined a significant effect of the treatment [$F(3,14) = 3.60$; $p = 0.04$]. Fisher's LSD test indicated that the group injected with IL-1β showed a reduction in levels of p-ERK2 30 min

after conditioning compared to the other experimental groups (SAL-SAL, D7,5-SAL and D7,5-IL-1β). The group DCS- IL-1β did not differ from control group.

4.7. Modulation by α-MSH of molecular changes induced by IL-1β after contextual fear conditioning

Our previous results indicated that treatment with α-MSH following IL-1β prevented the decrease in contextual fear memory (Gonzalez et al., 2009). In order to determine the mechanism involve in this effect we investigated the molecular pathways modulated by this neuropeptide. α-MSH (0.05 μg/0.25 μl) administration induced no changes in glutamate release after fear conditioning and failed to reverse the decrease induced by IL-1β (5 ng/0.25 μl) (data not shown). One way ANOVA showed a significant effect for the treatment [$F(3,14) = 6.48$; $p = 0.006$]. Newman Keuls test demonstrated that glutamate release was similar in the control group and group treated with α-MSH and statistically different in groups treated with IL-1β or IL-1β-α-MSH compared to the control group. It has been demonstrated that α-MSH, through MC4R, can activate MAPK ERK1/2 in different experimental models (Chai et al., 2006; Vongs et al., 2004). Injection of α-MSH (0.05 μg/0.25 μl) in dorsal hippocampus after treatment with IL-1β produced an increase in p-ERK2 30 min after fear conditioning, reverting the effect of IL-1β and restoring levels of control animals (Fig 8). One way ANOVA indicated a significant effect of the treatment [$F(3,23) = 5.79$; $p = 0.004$]. Fisher's LSD test demonstrated that IL-1β significantly reduced ERK2 phosphorylation compared to the other groups but there was no significant difference in p-ERK2 between groups treated with α-MSH, IL-1β and α-MSH or saline.

Barrientos et al. (2004) describe an increase of BDNF mRNA in dorsal hippocampus 120 min after contextual fear conditioning. Therefore, we measured BDNF protein expression in dorsal hippocampus 180 min after training. Concordantly with the bibliography

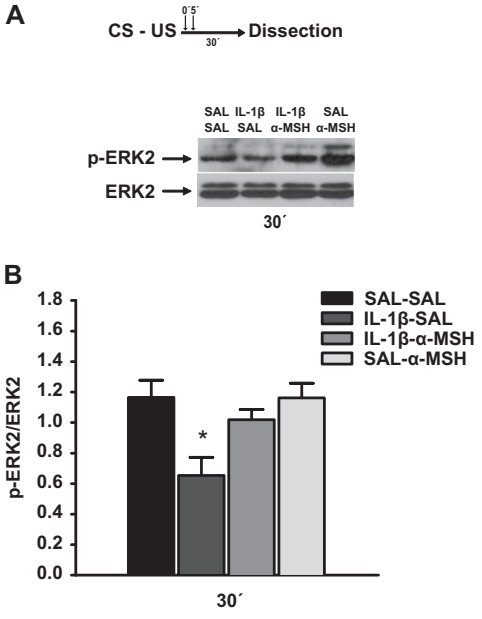

Fig. 8. Effect of α-MSH on ERK phosphorylation inhibition induced by IL-1β after contextual fear conditioning. Rats were treated in dorsal hippocampus immediately after training with saline or IL-1β (5 ng), 5 min later with saline or α-MSH and killed 30 min after conditioning. ERK2 phosphorylation was analyzed by western blot in total homogenates prepared from dorsal hippocampus. (A) Representative western blot of p-ERK2 and total ERK2 levels. (B) Data are expressed as mean ± S.E.M. of p-ERK2/ERK2 ratio. $n = 6–7$ per group. $^*p < 0.05$ versus other groups.

(Barrientos et al., 2004; Takei et al., 2012) the BDNF expression was lower in animals treated with IL-1β (5 ng/0.25 μl) after conditioning, compared with controls (Fig. 9). However, treatment with α-MSH (0.05 μg/0.25 μl) in dorsal hippocampus reversed the effect of IL-1β on BDNF expression 180 min after conditioning. The levels of BDNF expression in the group that received only α-MSH were similar to controls (Fig 9). One way ANOVA indicated a significant effect of the treatment [$F(3,12) = 4.09$; $p = 0.032$]. Fisher's LSD test demonstrated that IL-1β significantly reduced BDNF levels compared to control group and there were no significant differences in protein levels between groups treated with α-MSH-IL-1β and α-MSH or saline.

5. Discussion

The objective of this study was to identify cellular mechanisms induced by intrahippocampal administration of IL-1β that might lead to inhibition of memory consolidation. We previously demonstrated that IL-1β could interfere with contextual memory consolidation (Gonzalez et al., 2009). In the present work we determined that this effect was mediated by p38 activation in dorsal hippocampus. This could be a rapid presynaptic effect that would lead to a decrease of glutamate release. As a consequence, we observed a temporal delay in ERK phosphorylation and a reduction in BDNF levels after fear conditioning. We also established that treatment with α-MSH in dorsal hippocampus could restore levels of p-ERK and BDNF and, as a result, reverse the effects of IL-1β on memory consolidation.

NF-κB and p38 MAPK are activated by IL-1β in hippocampal astrocytes, but only p38 is activated in neurons (Srinivasan et al., 2004). Concordantly, inhibition of LTP induced by LPS and IL-1β was coupled with stimulation of p38 MAPK in hippocampus and entorhinal cortex an effect abrogated by the p38 inhibitor SB203580 (Kelly et al., 2003). In order to establish the participation of p38 in the effect of IL-1β on fear memory consolidation, we determined the activity of p38 after conditioning and found that the phosphorylation of this MAPK was increased in dorsal hippocampus in the presence of this cytokine. According to this result, administration of SB203580, a p38 inhibitor, reversed the impairment in memory consolidation induced by IL-1β. Levels of p-p38 in the control group remained constant at all the times studied, suggesting that this MAPK is not activated by fear conditioning. Also, administration of SB203580 did not modify the percent of freezing in control animals. Our results agreed with others that demonstrated that intrahippocampal infusion of SB203580 after training did not interfere with spatial memory in the Morris water test (Blum et al., 1999). Neither did SB203580 treatment affect LTP (Coogan et al., 1999).

In order to determine the effect of IL-1β in some of the preponderant molecular pathways involved in memory consolidation, we subsequently determined the effect of this cytokine on glutamate release, ERK phosphorylation and BDNF expression after contextual fear conditioning.

Our results demonstrated that the impairment induced by IL-1β on memory consolidation was coupled to inhibition of glutamate release. IL-1β was demonstrated to decrease glutamate release from hippocampal synaptosomes after LTP (Kelly et al., 2003; Vereker et al., 2000). Furthermore, the increase in KCl-stimulated glutamate release associated with LTP was attenuated in aged rats, in which IL-1β concentrations were elevated (O'Donnell et al., 2000), and in rats treated intracerebroventricularly with IL-1β (Vereker et al., 2000).

IL-1β-induced reduction of glutamate release could be related to its reported inhibitory effect on calcium channel activity in hippocampal neurons (Cunningham et al., 1996; Plata-Salaman and

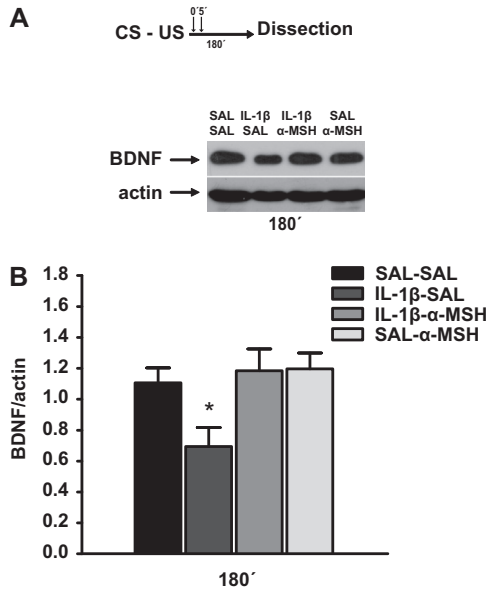

Fig. 9. Effect of α-MSH on impairment induced by IL-1β on the increase in BDNF levels after contextual fear conditioning. Animals received intra-hippocampal injections of saline or IL-1β (5 ng) immediately after training and 5 min later injections of saline or α-MSH (0.05 μg). Rats were killed 180 min after conditioning. BDNF levels were analyzed by western blot in total homogenates prepared from dorsal hippocampus. (A) Representative immunoblots of BDNF and total actin levels. (B) Data are expressed as mean ± S.E.M. of BDNF/actin ratio. $n = 4$ per group. $^{*}p < 0.05$ versus other groups.

Ffrench-Mullen, 1992). The inhibitory effect of IL-1β was rapid, which probably reflects the fact that this cytokine could affect calcium-dependent release, which occurs quickly following depolarization. Consequently, IL-1β-induced decrease of glutamate release might be secondary to the inhibitory effect on calcium influx previously reported.

Our results showed that SB203580 could partially reverse the effect of IL-1β on glutamate release during memory reconsolidation, indicating that p38 contributes to this effect. The p38 activation could regulate glutamate release through different mechanisms. MAPK could be localized both at pre-synaptic and post-synaptic levels in the hippocampus (Fiore et al., 1993; Jovanovic et al., 1996), and they have been demonstrated to regulate activation of proteins of the complex of fusion and ionic channels dependent on voltage (Jovanovic et al., 1996; Roberson et al., 1999; Varga et al., 2000).

Considering that our results suggest that memory impairment induced by IL-1β could be mediated by a decrease in glutamate release, the increment of glutamatergic transmission would reverse impairment of memory consolidation induced by the cytokine. This hypothesis was confirmed by treatment with DCS, a partial agonist of NMDA receptor that increases glutamatergic transmission. DCS was able to facilitate learning and memory in different paradigms (Kalisch et al., 2009; Richardson et al., 2004). The administration of DCS in a dose that did not affect fear conditioning "per se" reversed impairment of memory consolidation induced by IL-1β. This result suggests that the reduction of glutamate release induced by IL-1β after fear conditioning could be one of the crucial mechanisms involved in the effect of this cytokine on memory consolidation. It has been also demonstrated that cognitive deficits observed during hippocampal injuries, aging or dopaminergic neurodegeneration could be reversed by DCS administration (Billard and Rouaud, 2007; Wang et al., 2010).

ERK MAPK was shown to be phosphorylated in the hippocampus after LTP, an effect that was blocked, along with LTP, by pre-treatment with inhibitors of ERK activation (English and Sweatt, 1996). Furthermore, contextual fear conditioning induced ERK phosphorylation in hippocampus and inhibition of the activation of this MAPK reduced the response after fear conditioning (Sananbenesi et al., 2002; Sindreu et al., 2007). Our results demonstrated that ERK was activated 30 min after conditioning and that treatment with IL-1β produced a temporally different ERK activation.

Usually, activation of ERK after fear conditioning training is a consequence of NMDA activation by glutamate release (Athos et al., 2002; Chwang et al., 2006). Hence, the reduction of glutamate release induced by IL-1β could interfere with ERK phosphorylation. Concording with this proposition, DCS treatment reversed the reduction of ERK phosphorylation induced by IL-1β.

Molecular pathways activated as a result of a learning experience ultimately lead to activation of target genes and protein synthesis. BDNF is one of the more specific proteins involved in memory consolidation, and its expression increased in hippocampus after contextual fear conditioning (Hall et al., 2000). We also described that the administration of IL-1β decrease BDNF expression in dorsal hippocampus after fear conditioning. Our results concord with others that reported that the administration of IL-1β or the increase of cytokines in the CNS reduces the expression of BDNF in hippocampus after contextual learning (Barrientos et al., 2004; Takei et al., 2012).

We previously demonstrated that α-MSH, aside from its broad anti-inflammatory effects in the CNS, could reverse impairment in memory consolidation induced by IL-1β (Gonzalez et al., 2009). In this study we demonstrated that α-MSH administration in dorsal hippocampus after fear conditioning neither modified glutamate release nor could reverse inhibition induced by IL-1β of this process.

It has been reported that α-MSH could produce activation of ERK pathway in different experimental models. Thus, the agonist of MC4R, Melanotan II, increased ERK1/2 and CREB phosphorylation in vivo (Sutton et al., 2005). Furthermore, NDP-α-MSH, an α-MSH analogue, induced a dose dependent increase in ERK activation in the GT1-1 and CHO-K1 cells (Vongs et al., 2004). Moreover, ERK-1/2 participates in the NDP-α-MSH modulation of PPAR protein expression in astrocytes and microglia (Carniglia et al., 2013). Therefore, we also evaluated the effect of α-MSH on ERK activation after contextual fear conditioning. We showed that administration of α-MSH in dorsal hippocampus after treatment with IL-1β restored levels of p-ERK. Accordingly, injection of α-MSH prevents the reduction induced by IL-1β on BDNF expression in dorsal hippocampus. Other experimental models have demonstrated that the melanocortins can increase BDNF expression. MC4R activation was reported to regulate the eating behavior through BDNF release in hypothalamus (Xu et al., 2003). Also, NDP-α-MSH increased BDNF mRNA and protein in cultured astrocytes (Caruso et al., 2012).

In conclusion, IL-1β interferes in the process of consolidation of contextual fear memory, possibly through a presynaptic mechanism involving p38 MAPK activation in dorsal hippocampus. p38 phosphorylation produced a decrease in glutamate release and consequently a temporal delay in ERK activation and a reduction in BDNF expression during the consolidation process. These biochemical events could be the neurobiological substrate of the detrimental effect of IL-1β on memory consolidation. α-MSH reversed the effect of IL-1β by restoring levels of ERK phosphorylation and expression of BDNF. α-MSH may exert a beneficial modulatory role in preventing IL-1β effects on cognitive processes.

Acknowledgments

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