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Interleukin-1β-induced memory reconsolidation impairment is mediated by a reduction in glutamate release and zif268 expression and α-melanocyte-stimulating hormone prevented these effects

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ABSTRACT

The immune system is an important modulator of learning, memory and neural plasticity. Interleukin 1β (IL-1β), a pro-inflammatory cytokine, significantly affects several cognitive processes. Previous studies by our group have demonstrated that intrahippocampal administration of IL-1β impairs reconsolidation of contextual fear memory. This effect was reversed by the melanocortin alpha-melanocyte-stimulating hormone (α-MSH). The mechanisms underlying the effect of IL-1β on memory reconsolidation have not yet been established. Therefore, we examined the effect of IL-1β on glutamate release, ERK phosphorylation and the activation of the transcription factor zinc finger- 268 (zif268) during reconsolidation. Our results demonstrated that IL-1β induced a significant decrease of glutamate release after reactivation of the fear memory and this effect was related to calcium concentration in hippocampal synaptosomes. IL-1β also reduced ERK phosphorylation and zif268 expression in the hippocampus. Central administration of α-MSH prevented the decrease in glutamate release, ERK phosphorylation and zif268 expression induced by IL-1β. Our results establish possible mechanisms involved in the detrimental effect of IL-1β on memory reconsolidation and also indicate that α-MSH may exert a beneficial modulatory role in preventing IL-1β effects.

1. Introduction

It is now well established that the immune system, and in particular the pro-inflammatory cytokine interleukin-1β (IL-1β), can modulate brain functioning. When the immune system is activated by infection, injury or chronic inflammatory conditions, as well as by severe or chronic stressful conditions, glia and/or some neuron populations can secrete high levels of pro-inflammatory cytokines that produce detrimental effects on memory, neural plasticity and neurogenesis. Also, pathologies with increased peripheral levels of cytokines such as autoimmune diseases and after administration for therapeutic purposes, are associated with cognitive disturbance in humans, particularly with deficits in learning and memory (Yirmiya and Goshen, 2011).

New memories are transformed from an initially labile short-term memory state into a stable long-term memory over time by a process called consolidation (McGaugh, 2000). However, consolidated memories may again become labile and susceptible to disruption when reactivated. Therefore, retrieval of a previously consolidated memory may induce a new labile period during which the memory can be modified and this may require an active process to stabilize the memory again. This process has been called reconsolidation (Nader et al., 2000; Dudai, 2006). Its adaptive purpose might be to permit the integration of new information present at the time of retrieval into an updated memory. However, the reconsolidation process is not a simple reiteration of consolidation (Tronson and Taylor, 2007). Although both processes require de novo protein synthesis for memory persistence (Nader et al., 2000), it has been reported that the molecular mechanisms underlying these two processes within the hippocampus are different

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(Lee et al., 2004). Thus, brain-derived neurotrophic factor (BDNF) is selectively required for memory consolidation, whereas the transcription factor zinc finger-268 (zif-268) is selectively required for reconsolidation of contextual fear memory (Lee et al., 2004). Moreover, zif-268 is activated in the hippocampus by retrieval of a contextual fear memory (Hall et al., 2001). It has been proposed that signaling pathways activated during the reconsolidation process are induced by glutamate release. Glutamate participation was established using different antagonists of AMPA or NMDA receptors in different species and paradigms (Pedreira et al., 2002; Suzuki et al., 2004) Other studies showed that reconsolidation requires phosphorylation of GluR1 glutamate receptor subunits (Monfils et al., 2009). Stimulation of glutamate receptors produces activation of different kinases. Two of them, the extracellular signal-regulated kinase (ERK) and protein kinase A (PKA) have attracted particular interest due their well-established roles in reconsolidation.

The effect of IL-1β in memory consolidation has been extensively studied. It has been proposed that basal IL-1β levels are needed for memory consolidation, but that increased IL-1β levels induced by immune, physical, psychological challenge or even blockade of IL-1β signaling result in memory impairment (Pugh et al., 1998; Barrientos et al., 2002; Yirmiya and Goshen, 2011). However, recent studies in IL-1RI$^{-/-}$ mice showed no significant impairment in hippocampal dependent memory and learning, which may lead to re-evaluation of the role of endogenous IL-1 in hippocampal function (Murray et al., 2013).

We previously demonstrated that IL-1β interferes with contextual fear memory consolidation (Gonzalez et al., 2009, 2013). The effects of immune activation or cytokines on memory reconsolidation have been studied far less (Machado et al., 2010; Kranjac et al., 2012). We recently shown that IL-1β infused directly into the hippocampus may also have a detrimental effect on reconsolidation of contextual fear memory (Machado et al., 2010). In other experimental models IL-1β was shown to modify some of the signaling molecules critically involved in memory reconsolidation. In these reports, IL-1β reduced glutamate release during LTP (Kelly et al., 2003), decreased ERK phosphorylation during memory consolidation (Gonzalez et al., 2013) and in activated microglia in culture (Saud et al., 2005) and also decreased zif268 expression in human epidermal keratinocytes (Lukiw et al., 1998). However, the molecular mechanism involved in the effect of IL-1β on memory reconsolidation has not yet been established.

Melanocortins, endogenous peptides produced by post-translational processing of pro-opiomelanocortin (POMC), have been implicated in multiple processes in the central nervous system (CNS) including immunomodulatory effects (Catania et al., 2010; Caruso et al., 2014). Melanocortins exert actions through five different G protein-coupled receptors (MC1/MC5). MC3 and MC4 receptors are predominantly expressed throughout the CNS including the hippocampus (Muceniece and Dambrova, 2010). α-MSH through activation of MC4R reverses the effect of IL-1β on consolidation (Gonzalez et al., 2009) and reconsolidation of a contextual fear memory (Machado et al., 2010). It has been reported that α-MSH could produce activation of ERK pathway in different experimental models, in vivo (Sutton et al., 2005) in CHO-K1 cells (Vongs et al., 2004), and in astrocytes and microglia (Carniglia et al., 2013). α-MSH can also increase zif268 expression in hippocampus, improving learning and memory after cerebral ischemia (Giuliani et al., 2009). Moreover, α-MSH induced over-expression of zif268 and improved cognitive functions in an Alzheimer's disease model through MC4R activation (Giuliani et al., 2014). Therefore, the molecular mechanism involved in memory reconsolidation could be modified by IL-1β and/or α-MSH to produce their effects.

The objective of this study was to identify molecular mechanisms induced by intrahippocampal administration of IL-1β that might lead to impairment of memory reconsolidation and also to evaluate the modulatory role of α-MSH.

Pro-inflammatory cytokines mediate cognitive impairments associated with several neuropsychiatric diseases and neurodegenerative disorders. Consequently it is of relevant importance to study signaling pathways involved in the detrimental effects of cytokines on memory processes. Also importantly, the immunomodulatory effects of melanocortins propose them as potentially useful in the treatment of undesirable central effects of cytokines.

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 (Res. 875/2014). The number of animals used as well as their suffering was kept to the minimum necessary for the goals of this study.

2.2. Surgery and drug infusion procedures

Animals were anesthetized with an intraperitoneal (ip) injection of ketamine hydrochloride (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): −3.2 mm; lateral (L): ±2.0 mm; dorso ventral (DV): −1.8 mm according to the rat brain atlas (Paxinos and Watson, 2007). Cannulas were fixed to the skull with a screw and dental acrylic. 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) and α-MSH (NeoMPS, France). The rrIL-1β was dissolved in sterile 0.9% saline containing 0.01% bovine serum albumin and stored in aliquots at −70 °C. The solution of 0.01% bovine serum albumin did not interfere with memory processes and did not cause any disturbance in tissue surrounding the injection. α-MSH were dissolved in sterile saline (0.9% sodium chloride) and stored in aliquots at −20 °C. 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). Sterile saline (SAL) (0.25 μl) was used for control injections.

2.4. 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.

2.4.1. Apparatus

The conditioning chamber was made of white acrylic ($44 \times 22 \times 20$ 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. The chamber was placed in a room illuminated by a 2.5 W 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 9:00 and 14:00 h with experimenters unaware of the treatment condition.

2.4.2. Procedure

Contextual fear conditioning (training): consisted in placing the rat individually in the conditioning chamber where it was left undisturbed for a 4 min acclimatization period (pre-shock period). After this period, rats received three unsignaled footshocks (0.68 mA; 2 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.

Re-exposure session (reactivation): twenty-four hours after training, animals were re-exposed to the training context without shocks for 3 min.

In order to determine changes in molecular mechanisms involved in memory reconsolidation (such as glutamate release, calcium influx, ERK2 phosphorylation or zif268 expression), the animals received the different treatments after reactivation session, were killed at different times depending on the experiment, and dorsal hippocampus was dissected as soon as possible.

2.5. Glutamate release

2.5.1. Preparation of hippocampal synaptosomes

The animals were killed by decapitation 10 min after reactivation. The dorsal hippocampus was dissected as previously described and synaptosomes were purified on discontinuous Percoll gradients as described previously (Gonzalez et al., 2013). Briefly, 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.

2.5.2. 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 (Gonzalez et al., 2013). Briefly, synaptosomal pellets were resuspended in HEPES buffer medium and incubated in a stirred and thermostated cuvette kept 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 adding 4 nmol of glutamate at the end of each assay. Data points were obtained at 1-s intervals.

2.6. Cytosolic Ca^{2+} measurements using Fluo-3AM

Synaptosomes (0.4 mg/ml) were pre-incubated in HEPES buffer medium in the presence of 1 mM Fluo-3AM for 30 min at 37 °C in a stirred test tube. After Fluo-3AM loading, synaptosomes were centrifuged for 30 s at 5000*g*. The synaptosomal pellets were resuspended in HEPES buffer medium and the synaptosomal suspension was stirred in a thermostated cuvette in a FluoroMax-P Horiba Jobin Yvonspectrofluorimeter. $CaCl_2$ (1.2 mM) was added after 3 min, and 10 min later, 3 mM 4AP was added. Fluorescence data were accumulated at 3-s intervals (excitation wavelengths at 506 and emission wavelength at 530 nm). Calibration procedures were performed as described (Grynkiewicz and Poenie, 1985) using 0.1% sodium dodecyl sulfate (SDS) to obtain maximal fluorescence with Fluo-3AM saturation with Ca^{2+}, followed by 10 mM EGTA (Tris buffered) to obtain minimum fluorescence in the absence of any Fluo-3AM/Ca^{2+} complex. Cytosolic free Ca^{2+} concentration was calculated as described previously for quin2 (Tsien et al., 1982), but considering the F_{min} factor as the basal levels of calcium before 4AP and using a KD for Fluo-3AM of 400 nM.

2.7. Western blotting

Animals were killed by decapitation at different times depending on the experiment: 15, 30 and 60 min after retrieval for ERK and 0, 30 and 60 min after retrieval for zif268 determination.

The brains were 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). The hippocampus tissue was 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 (40 μg/lane) were separated by 10% SDS–PAGE and transferred onto polyvinylidene fluoride membrane (BioRad).

For detection of phosphorylated ERK and zif268, 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 polyclonal anti-Egr1 (zif268) (1:500, Santa Cruz) respectively. Blots were then incubated with anti-rabbit conjugated to horseradish peroxidase (1:2500, Jackson) for 120 min at room temperature (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 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); 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 the ratio of phospho-ERK2/total ERK2, zif268/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 p-ERK1 signals were often too faint and inconsistent to be accurately analyzed.

2.8. 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 test. When no interaction was found contrast analysis was run (Montgomery, 2001). When only two groups were compared, Student's *t*-test was used. In all tests, differences with p values $\leqslant 0.05$ were considered statistically significant.

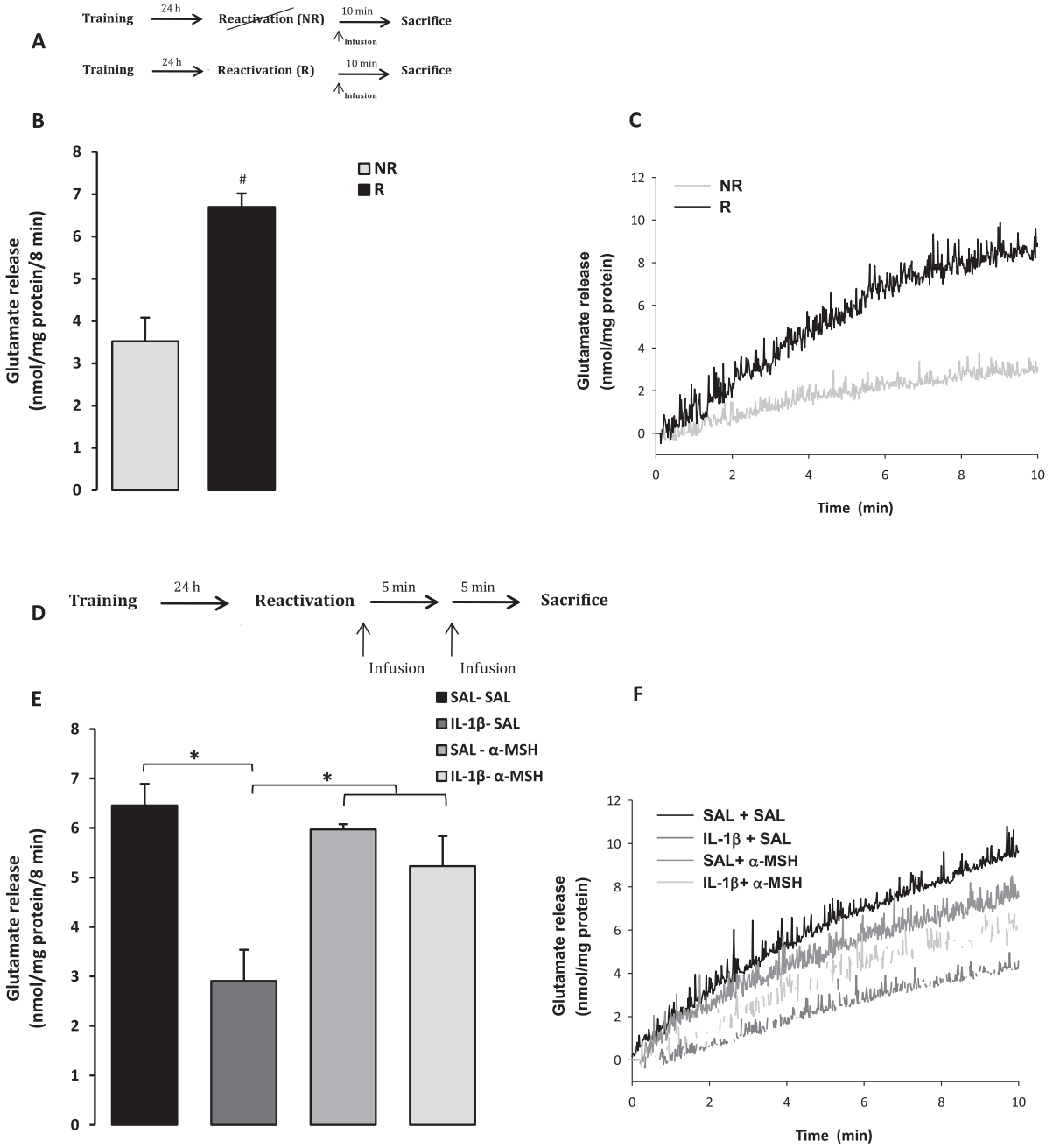

Fig. 1. IL-1β reduced glutamate release after memory reactivation and α-MSH prevented this effect. Glutamate release induced by 4-AP was assessed in synaptosomes prepared from dorsal hippocampus. Synaptosomes were purified on discontinuous Percoll gradients. (A and D) Schematic representation of experimental procedures. (B) Animals were treated in dorsal hippocampus with saline immediately after memory reactivation (R) or without reactivation (NR). (E) After reactivation of a contextual fear memory rats were treated with SAL + SAL, IL-1β + SAL, SAL + α-MSH or IL-1β + α-MSH. Values registered after 8 min were expressed as mean ± S.E.M., $n = 4–5$, $^{\#}p < 0.005$ respect NR $^{*}p < 0.05$ vs other groups. (C and F) Traces are representative of independent experiments.

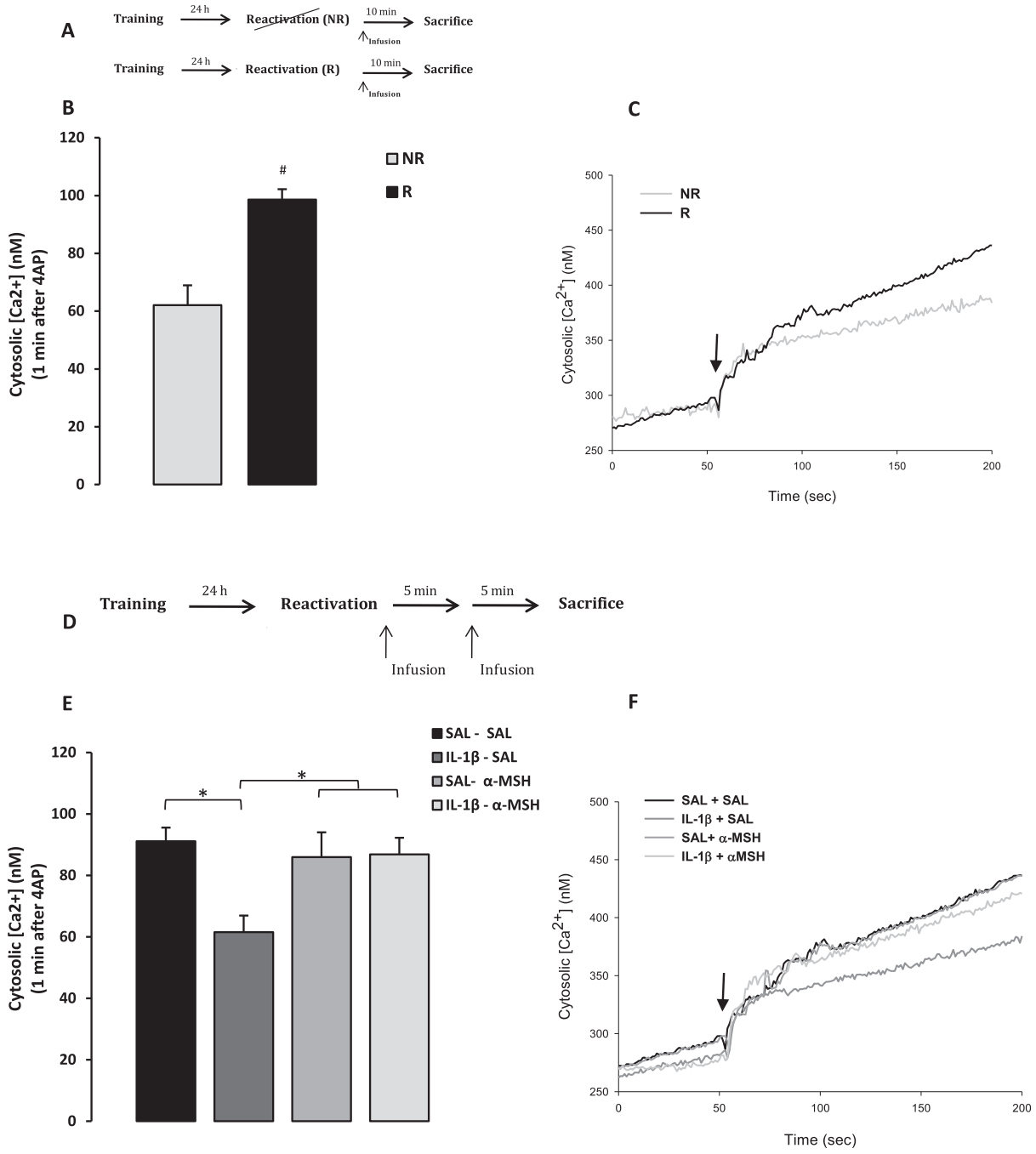

Fig. 2. L-1β reduced cytosolic calcium influx and co-treatment with α-MSH prevented this effect. Calcium influx was assessed in synaptosomes prepared from dorsal hippocampus. Synaptosomes (0.4 mg/ml) were incubated as described in experimental procedures and cytosolic [Ca^{2+}] was monitored using Fluo-3AM. Voltage-dependent Ca^{2+} influx was evoked by adding 4-aminopyridine (4AP) (arrow). (A and D) Schematic representation of experimental procedures. (B). Animals were treated in dorsal hippocampus with saline immediately after memory reactivation (R) or without reactivation (NR) (E) Animals were treated after reactivation of a contextual fear memory with SAL + SAL, IL-1β + SAL, SAL + α-MSH or IL-1β + α-MSH. Values registered 1 min after 4AP were expressed as mean ± S.E.M. $^{\#}p < 0.005$ respect NR group, $n = 3–5$, $^{*}p < 0.05$ vs other groups, $n = 4–5$. (C and F) Traces are representative of independent experiments.

3. Results

3.1. IL-1β reduced glutamate release after fear memory reactivation and α-MSH prevented this effect

In order to determine glutamate release induced by memory reconsolidation in our experimental model, two different groups of animals were trained. In one group, memory was reactivated (R group, re-exposed to the context) and in the other group, memory was not reactivated (NR group, i.e. no re-exposure to the context). We injected saline solution in both groups: in the R group immediately after memory reactivation and in the other group 24 h after training; then, glutamate release from hippocampal synaptosomes was determined in both groups. Memory reconsolidation produced an increase in glutamate release from dorsal hippocampal synaptosomes (Fig. 1B). Student *t* test indicated that glutamate release from R group was significantly higher than in the NR group ($t = -5.23$; df = 7; $p < 0.005$).

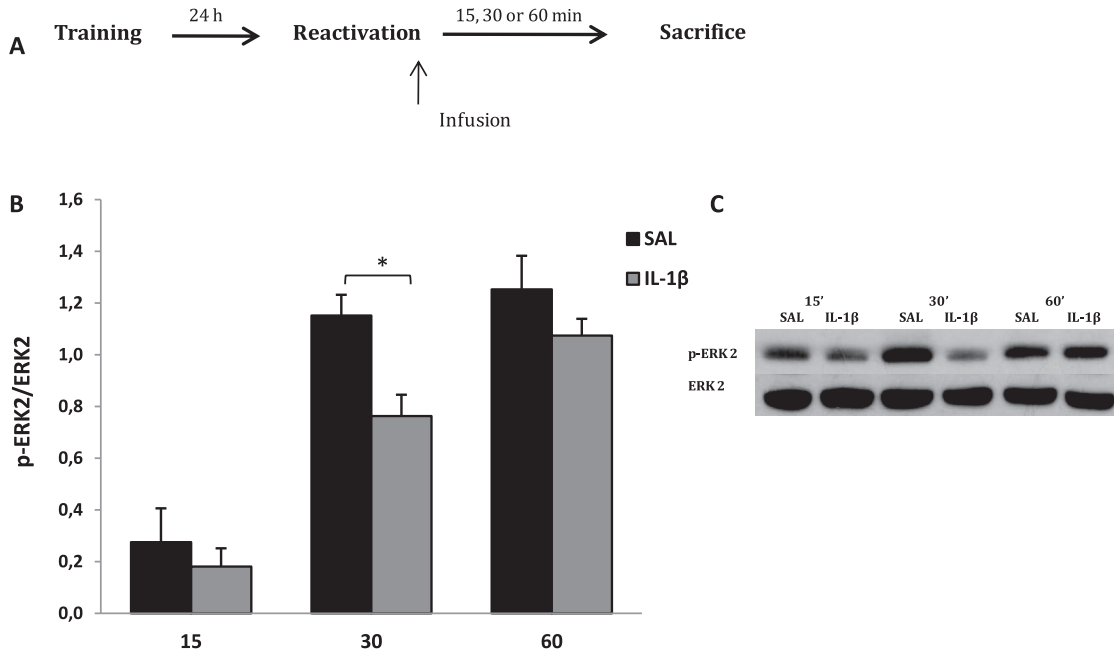

Fig. 3. ERK2 phosphorylation increased after reactivation of a contextual fear memory and IL-1β reduced it. ERK2 phosphorylation was analyzed by Western blot in total homogenates prepared from the dorsal hippocampus (A) Schematic representation of experimental procedures. (B) Animals were injected immediately after retrieval of a contextual fear memory with SAL or IL-1β and killed 15, 30 or 60 min later. Data are expressed as the mean of p-ERK2/ERK2 ratio ± S.E.M, $n = 3–5$, $^{*}p < 0.05$. (C) Representative Western blot of p-ERK2 and total ERK2 levels.

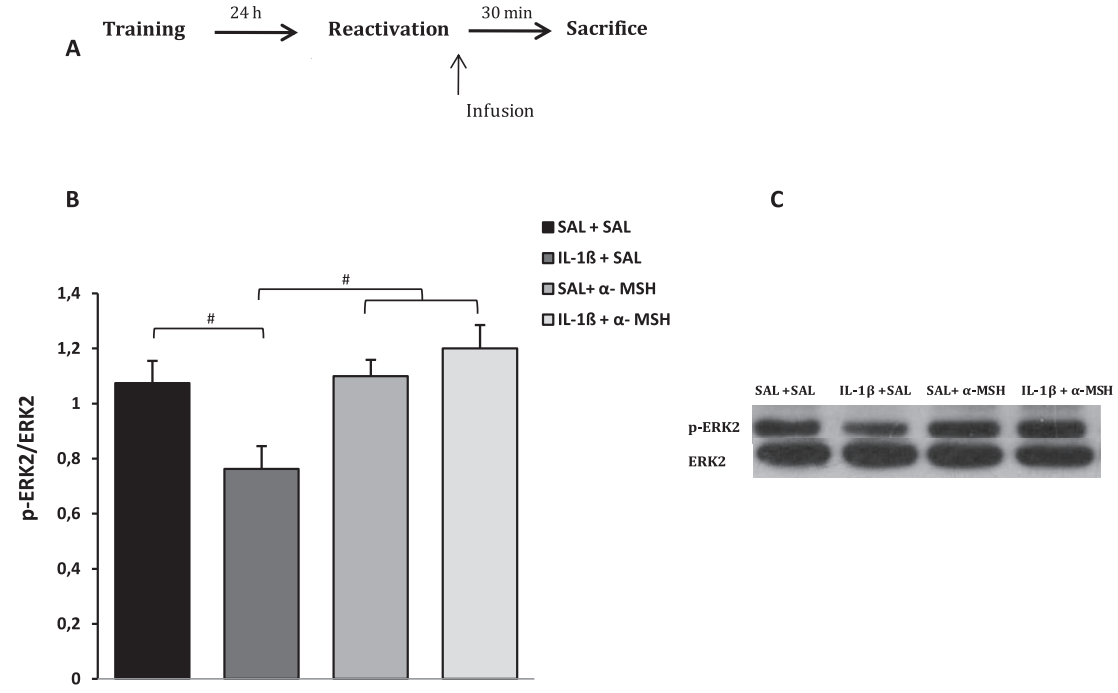

Fig. 4. IL-1β reduced ERK2 phosphorylation during memory reconsolidation and α-MSH prevented this effect. ERK2 phosphorylation was analyzed by Western blot in total homogenates prepared from the dorsal hippocampus. (A) Schematic representation of experimental procedures. (B) Animals were injected immediately after retrieval of a contextual fear memory with SAL + SAL, IL-1β + SAL, SAL + α-MSH or IL-1β + α-MSH and killed 30 min later. Data are expressed as the mean of p-ERK2/ERK ratio ± S.E.M, $n = 5–6$, $^{\#}p < 0.05$ vs other groups. (C) Representative Western blot of p-ERK2 and total ERK2 levels.

We also determined glutamate release from synaptosomes obtained from dorsal hippocampus of rats treated with saline, IL-1β and/or α-MSH immediately after reactivation. Treatment with IL-1β reduced glutamate release compared to control group (Fig. 1E). Our previous results indicated that treatment with α-MSH following IL-1β prevented the decrease in contextual fear memory reconsolidation (Machado et al., 2010). In order to determine the mechanism involve in this effect we investigated the effect of α-MSH on glutamate release. α-MSH administration induced no changes in glutamate release during memory reconsolidation at the dose tested, but prevented the decrease induced by IL-1β (Fig. 1E). Analysis of endogenous glutamate release by one

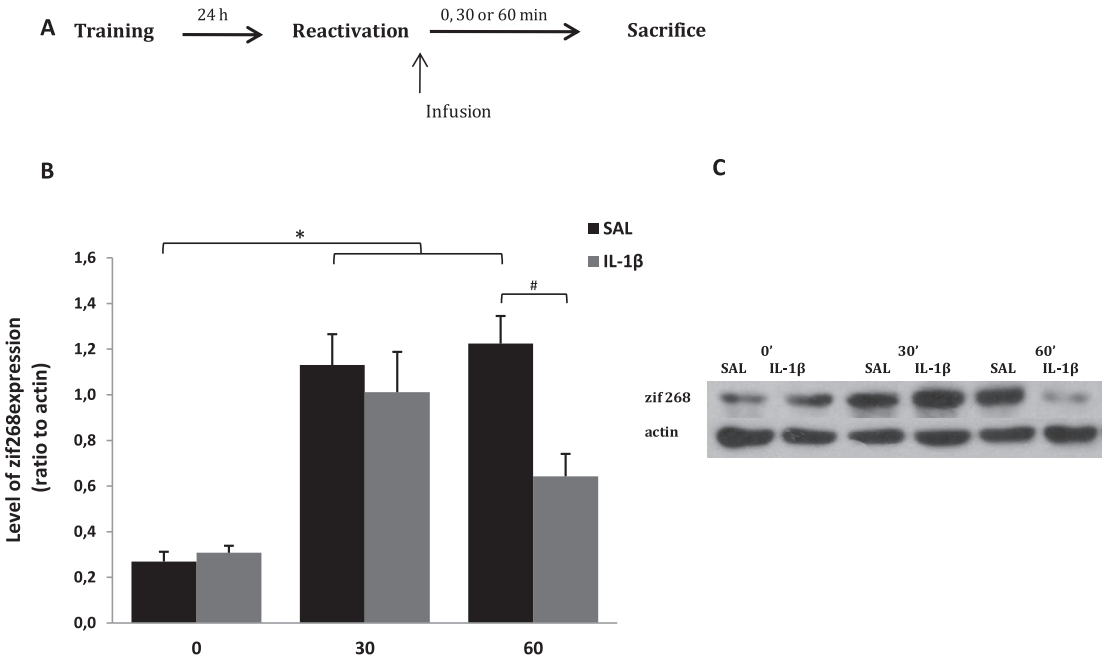

Fig. 5. Zif268 expression increased after reactivation of a contextual fear memory and IL-1β reduced it. Zif268 expression was analyzed by Western blot in total homogenates prepared from the dorsal hippocampus. (A) Schematic representation of experimental procedures. (B) Animals were injected immediately after retrieval of a contextual fear memory with SAL or IL-1β and killed 0, 30 or 60 min later. Data are expressed as the mean of zif268/actin ratio ± S.E.M. $n = 5–6$, $^{\#}p < 0.05$, $^{*}p < 0.005$ vs SAL 0′. (C) Representative Western blot of zif268 and actin levels.

way ANOVA revealed a significant effect of treatment [$F(3,14) = 9.54$; $p < 0.005$]. The post hoc analysis by Newman–Keuls test indicated that glutamate release from the IL-1β group was significantly different from the other groups ($p < 0.05$).

3.2. IL-1β reduced calcium influx during memory reconsolidation and α-MSH prevented this effect

The effect of IL-1β on glutamate release could be related to diminish Ca^{2+} influx in the presynaptic terminal induced by the cytokine. First, Ca^{2+} influx was measured in synaptosomes obtained from dorsal hippocampus of NR animals or after memory reactivation (R group), treated with saline. Memory reconsolidation produced an increase of cytosolic Ca^{2+} influx. Student t test showed a significant difference ($t = 5.23$; df = 6; $p = 0.003$) in the cytosolic Ca^{2+} influx between NR and reactivated groups (Fig. 2B).

IL-1β reduced Ca^{2+} influx during memory reconsolidation (Fig. 2E). Also, α-MSH administration prevented the effect of IL-1β (Fig. 2E). Analysis of cytosolic Ca^{2+} influx by one way ANOVA revealed a significant effect of treatment [$F(3,16) = 7.05$; $p < 0.005$]. Post hoc analysis by Newman–Keuls test indicated that calcium influx in synaptosomes obtained from IL-1β group was significantly different from the other groups ($p < 0.05$).

3.3. IL-1β reduced ERK2 phosphorylation during memory reconsolidation and α-MSH prevented this effect

We next examined ERK2 phosphorylation in dorsal hippocampus after contextual fear memory reactivation. Western immunoblotting analysis was run in different groups of rats that were trained and re-exposed to the context for memory reactivation, subsequently receiving saline or IL-1β before being killed 15, 30 or 60 min after reactivation.

Memory reconsolidation induced changes in ERK2 phosphorylation over time as indicated by two-way ANOVA [$F(2,28) = 41.31$; $p < 0.001$]. This analysis also showed a significant effect of treatment [$F(2,28) = 5.67$; $p < 0.05$] but there was no effect on interaction between treatment and time [$F(2,28) = 1.29$; $p = 0.29$] (see Fig. 3).

The ERK2 phosphorylation pattern seems to be similar in both experimental groups. However, we observed a reduction in p-ERK2 level in the IL-1β group compared to controls 30 min after reactivation. To compare these groups we ran a contrast within the ANOVA (Montgomery, 2001) which shows a significant difference between them [$F(1,10) = 7.52$; $p < 0.05$].

We next evaluated the effect of α-MSH on the decrease in ERK2 phosphorylation produced by IL-1β 30 min after reactivation. Injection of α-MSH into dorsal hippocampus after treatment with IL-1β produced an increase in p-ERK2 30 min after fear memory retrieval, preventing the effect of IL-1β and restoring levels of control animals (Fig. 4B). One way ANOVA indicated a significant effect of the treatment [$F(3,22) = 4.54$; $p < 0.05$] and Newman–Keuls test demonstrated that IL-1β significantly reduced ERK2 phosphorylation compared to the other groups ($p < 0.05$), although there was no significant difference in p-ERK2 between groups treated with SAL + SAL, SAL + α-MSH and IL-1β + α-MSH.

3.4. IL-1β reduced zif268 expression during memory reconsolidation and α-MSH prevented this effect

Memory reconsolidation is associated specifically with zif268 expression (Lee et al., 2004). Therefore, we measured zif268 expression in dorsal hippocampus at several intervals after retrieval of fear conditioning. Different groups of animals were exposed to the context 24 h after conditioning and immediately injected in hippocampus with SAL or IL-1β and killed immediately, 30 and 60 min after retrieval. Results showed increased zif268 expression in dorsal hippocampus at 30 min, which lasted at least up to 60 min after reactivation in the control group. In animals treated with IL-1β, zif268 expression was reduced after 60 min (Fig. 5B). Two way ANOVA showed significant interaction between treatment and time [$F(2,28) = 3.61$; $p = 0.04$]. Newman–

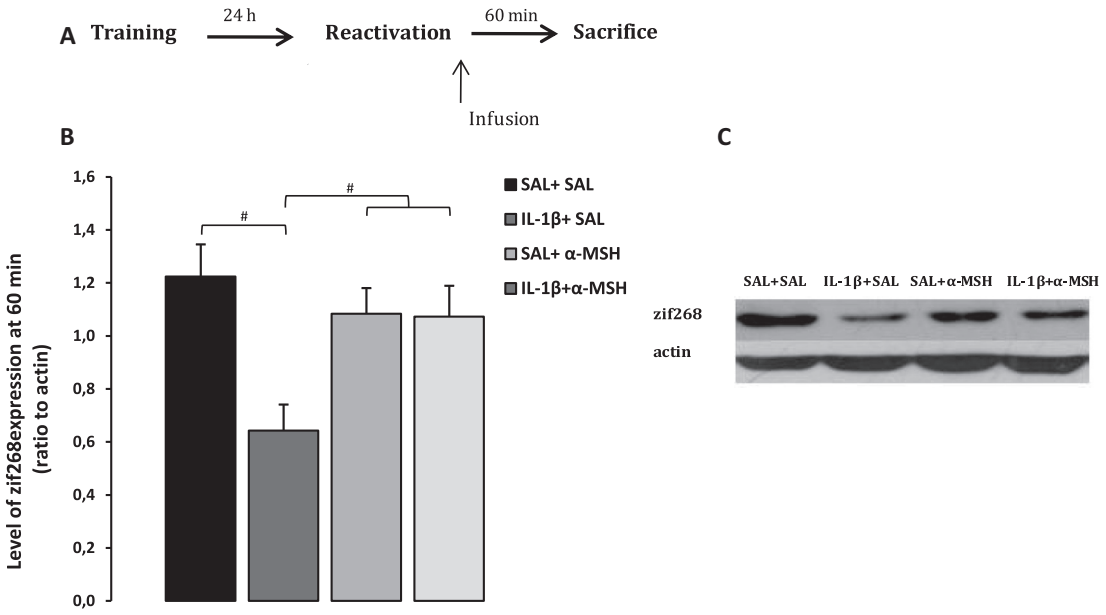

Fig. 6. IL-1β reduced zif268 expression during memory reconsolidation and α-MSH prevented this effect. Zif268 expression was analyzed by Western blot in total homogenates prepared from the dorsal hippocampus. (A) Schematic representation of experimental procedures. (B) Rats were injected immediately after retrieval of a contextual fear memory with SAL + SAL, IL-1β + SAL, SAL + α-MSH or IL-1β + α-MSH and killed 60 min later. Data are expressed as the mean of zif268/actin ratio ± S.E.M. $n = 6–7$, $^{\#}p < 0.05$ vs the other groups. (C) Representative Western blot of zif268 and actin levels.

Keuls test indicated that zif268 expression significantly increased 30 and 60 min after memory reactivation compared to time 0 in control groups ($p < 0.005$) and that this increase was not observed at 60 min in animals treated with IL-1β, zif268 expression levels being significantly lower than in controls at that time. Treatment with α-MSH in dorsal hippocampus also prevented the effect of IL-1β on zif268 expression 60 min after conditioning (Fig 6B). The levels of Zif268 expression in the group that received only α-MSH were similar to controls (Fig. 6B). One way ANOVA indicated a significant effect of the treatment [$F(3,21) = 5.05$; $p = 0.0086$]. Newman–Keuls test demonstrated that IL-1β significantly reduced zif268 expression compared to control group ($p < 0.05$) and that there were no significant differences between groups treated with SAL + SAL, SAL + α-MSH and IL-1β + α-MSH.

4. 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 reconsolidation. We previously demonstrated that IL-1β could interfere with contextual memory reconsolidation and that α-MSH reverses this effect (Machado et al., 2010). In the present work we determined that the effect of IL-1β was mediated by a decrease of glutamate release that could be related to diminished calcium concentration in the presynaptic terminal. We also observed a reduction in ERK phosphorylation and zif268 levels after memory retrieval. Treatment with α-MSH in dorsal hippocampus could restore glutamate release, Ca^{2+} concentration, levels of p-ERK and zif268 and, as a consequence, prevented the effect of IL-1β on memory reconsolidation as previously demonstrated.

We determined an increase in glutamate release and Ca^{2+} influx after memory retrieval. We also observed changes in ERK activation and an increase in zif268 expression.

Although molecular mechanisms involved in memory reconsolidation have been extensively studied, different experimental models were used (for review, see Tronson and Taylor, 2007). Here we analyzed, in the same model, i.e. during fear memory reconsolidation, some of the relevant molecules involved in the memory reconsolidation signaling cascade.

Our results demonstrated that IL-1β reduced glutamate release, suggesting that IL-1β effect on memory reconsolidation was coupled to this inhibition. It has been established that IL-1β decreases glutamate release from hippocampal synaptosomes after LTP (Vereker et al., 2000; Kelly et al., 2003) and that 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); also in rats treated intracerebroventricularly with IL-1β (Vereker et al., 2000). Moreover, IL-1β decreases glutamate release during memory consolidation (Gonzalez et al., 2013).

Modification of ion channels allows integration of many types of cellular responses to influence neuronal activity, with important implications for plasticity and cognitive function. Experimental evidence has demonstrated that ion channels are targeted by cytokines, which can modulate their function (Viviani et al., 2007; Viviani and Boraso, 2011). Specifically, IL-1β has been reported to inhibit voltage gated Ca^{2+} channels (Plata-Salamán and Ffrench-Mullen, 1992, 1994; MacManus, 2000). Thus, IL-1β-induced inhibition of voltage gated Ca^{2+} channels may be related to its ability to reduce neurotransmitter release (Murray et al., 1997). In concordance with this, our results showed a decrease in intrasynaptosomal Ca^{2+} concentration after treatment with IL-1β. Consequently, IL-1β-induced decrease of glutamate release might be secondary to the inhibitory effect on calcium influx, and could reflect an effect on physiological exocytotic vesicular release.

During memory reconsolidation ERK phosphorylation is induced in hippocampus. 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 during memory reconsolidation.

Moreover, some cellular processes are specific to reconsolidation but differ from those activated following memory acquisition

or consolidation, indicating that the mnemonic processes underlying consolidation and reconsolidation differ. Zif268, one of the specific proteins involved in memory reconsolidation, showed increased expression in hippocampus during this process. Our results demonstrated an increase of zif268 expression in hippocampus. We also described that IL-1β administration decreased zif268 expression in dorsal hippocampus during memory reconsolidation.

It was also reported that peripheral administration of LPS in mice produced deficits in both memory consolidation and reconsolidation (Kranjac et al., 2012). However, LPS-induced effects on memory reconsolidation processes in this experimental model seem to be mediated by a zif-268-independent mechanism. No changes in zif-268 mRNA expression were found in cortex or hippocampus 4 h after peripheral LPS administration. Possible explanations to this discrepancy between the two studies may be the variation in species (mice vs rats) and/or the route of administration (i.p. vs intra-hippocampus). Another important difference is that we injected specifically IL-1β instead of LPS, which in turn elevated IL-1β and other cytokine levels in hippocampus, so that it is difficult to compare the effective doses administered in these two studies. Finally, perhaps the most important factor is the time after reactivation at which zif-268 was measured. This is an early transcription factor and is activated soon during memory reconsolidation (Tronson and Taylor, 2007). Also, it was reported that following a single intracerebroventricular injection of LPS into the lateral ventricle of mice, zif-268 expression was significantly lower in cortex, but not in hippocampus (Bonow et al., 2009). In this report mice were not trained in any behavioral protocol. We measured the effect of IL-1β injection in hippocampus specifically after memory retrieval, when memory is undergoing reconsolidation and zif-268 expression is increased.

Our previous results indicated that treatment with α-MSH following IL-1β administration prevented the decrease in contextual fear memory reconsolidation (Machado et al., 2010). In order to determine the mechanism involved in this effect we investigated molecular pathways modulated by this neuropeptide. We found that α-MSH administration in the hippocampus during memory reconsolidation prevented the effect of IL-1β on exocytotic glutamate release, probably as a consequence of normalizing Ca^{2+} concentration. It has been demonstrated that L-type Ca^{2+} currents could be modulated by a wide variety of hormones and neurotransmitters in smooth, skeletal, and cardiac muscle, in endocrine cells, and in neurons (Armstrong et al., 1991; Catterall, 2000). Upon binding to melanocortin receptors, MSH can induce cAMP increase and PKA activation, and it has been demonstrated that elevation of intracellular cAMP and protein phosphorylation mediated voltage-gated Ca^{2+} channel modulation and increase of Ca^{2+} influx (Bean et al., 1984). On the other hand, IL-1β-induced decrease of glutamate release could be mediated by p38 activation. MAPKs 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 shown to regulate activation of proteins of the complex of fusion and ionic channels dependent on voltage (Jovanovic et al., 1996; Varga, 2000). α-MSH decreased p38 phosphorylation in other inflammatory experimental models (Yoon et al., 2003; Smalley and Eisen, 2000). Hence, α-MSH could also modulate molecular pathways induced by IL-1β to restore the Ca^{2+} concentration reduced by this cytokine.

It has been reported that α-MSH produces 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, a synthetic melanocortin analog with long-lasting biological activity, induced a dose dependent increase in ERK activation in GT1-1 and CHO-K1 cells (Vongs et al., 2004). Therefore, we also evaluated the effect of α-MSH on ERK activation during memory reconsolidation. We showed that administration of α-MSH in dorsal hippocampus after treatment with IL-1β restored levels of p-ERK. Also, injection of α-MSH prevents the reduction induced by IL-1β on zif268 expression in dorsal hippocampus. Other experimental models have demonstrated that melanocortins can increase zif268 expression. Zif-268 is rapidly induced as transcription factor by a variety of stimuli including ischemia. Melanocortins promote functional recovery from ischemic stroke and Zif268 gene is involved in this effect (Giuliani et al., 2009). NDP-MSH can also induce neurogenesis after cerebral ischemia and zif-268 is upregulated in hippocampus as well (Giuliani et al., 2011). Thus, treatment of ischemic stroke with melanocortins is associated with an over-expression of zif-268, promoting neuronal recovery and neurogenesis (Giuliani et al., 2009, 2011; Spaccapelo et al., 2011). Also, melanocortins protect against progression of Alzheimeŕs disease, an effect associated with hippocampus zif-268 over-expression (Giuliani et al., 2014).

In conclusion, IL-1β interferes in the process of reconsolidation of contextual fear memory, possibly through a presynaptic mechanism involving Ca^{2+}-dependent exocytotic glutamate release in dorsal hippocampus. As a consequence of reduction in glutamate release, a temporal delay in ERK activation and a reduction in zif268 expression were also observed during the reconsolidation process. These biochemical events could clarify neurobiological substrates involved in the detrimental effect of IL-1β on memory reconsolidation. Understanding molecular mechanisms involved in the effect of IL-1β on reconsolidation could provide new insights into the participation of this cytokine in normal cognitive function and in neurodegenerative disorders characterized by elevated levels of cytokines in specific areas of the central nervous system. Moreover, α-MSH prevented the effect of IL-1β exerting a beneficial modulatory role in preventing IL-1β effects on cognitive processes. This effect, together with its very low toxicity, raises the possibility that it might be useful in the treatment of undesirable central effects of cytokines during inflammatory processes.

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