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IL-1β reduces GluA1 phosphorylation and its surface expression during memory reconsolidation and α-melanocyte-stimulating hormone can modulate these effects

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ABSTRACT

Pro-inflammatory cytokines can affect cognitive processes such as learning and memory. Particularly, interleukin-1β (IL-1β) influences hippocampus-dependent memories. We previously reported that administration of IL-1β in dorsal hippocampus impaired contextual fear memory reconsolidation. This effect was reversed by the melanocortin alpha-melanocyte-stimulating hormone (α-MSH). Our results also demonstrated that IL-1β produced a significant decrease in glutamate release from dorsal hippocampus synaptosomes after reactivation of the fear memory. Therefore, we investigated whether IL-1β administration can affect GluA1 AMPA subunit phosphorylation, surface expression, and total expression during reconsolidation of a contextual fear memory. Also, we studied the modulatory effect of α-MSH. We found that IL-1β reduced phosphorylation of this subunit at Serine 831 and Serine 845 60 min after contextual fear memory reactivation. The intrahippocampal administration of IL-1β after memory reactivation also induced a decrease in surface expression and total expression of GluA1. α-MSH prevented the effect of IL-1β on GluA1 phosphorylation in Serine 845, but not in Serine 831. Moreover, treatment with α-MSH also prevented the effect of the cytokine on GluA1 surface and total expression after memory reactivation. Our results demonstrated that IL-1β regulates phosphorylation of GluA1 and may thus play an important role in modulation of AMPAR function and synaptic plasticity in the brain. These findings further illustrate the importance of IL-1β in cognition processes dependent on the hippocampus, and also reinforced the fact that α-MSH can reverse IL-1β effects on memory reconsolidation.

1. Introduction

Newly formed memories are initially labile but become progressively consolidated over time (McGaugh, 2000). However, consolidated memories may again become labile and susceptible to disruption when retrieved in the right conditions (Nader et al., 2000; Tronson and Taylor, 2007). Therefore, retrieval of a previously consolidated memory may induce a new labile period during which the memory can be modified. The active process that stabilizes the memory again has been called reconsolidation (Dudai, 2006; Nader et al., 2000). During this labile state, the memory could be enhanced or disrupted. Pharmacological intervention immediately after or in the first hours after retrieval disrupt normal reconsolidation of memory and can result in a weakened or strengthened memory depending on the type of intervention used (Tronson and Taylor, 2007). Reconsolidation is a complex process that requires de novo protein synthesis and induces synaptic changes. Synaptic plasticity can be regulated at the presynaptic level by altering neurotransmitter release or at postsynaptic level by changing the density, types, and properties of neurotransmitter receptors. These processes, including trafficking of ionotropic glutamate receptors, have been studied in the context of memory reconsolidation (Rao-ruiz et al., 2011; Jarome et al., 2012; Yu et al., 2013; Fukushima et al., 2014; Lopez et al., 2015).

Changes in the numbers and composition of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
IL-1β	interleukin 1-β
LTP	long term potentiation
MCR	melanocortin receptor
α-MSH	α-melanocyte-stimulating hormone

localized at the postsynaptic membrane are essential for synaptic plasticity. AMPA receptors are tetrameric complexes with four subunits designated as GluA1–4 (Rosenmund et al., 1998; Wenthold et al., 1996). The most common forms in hippocampus include GluA1/2 and GluA2/3 heteromers as well as GluA1 homomers (Craig et al., 1993; Lu et al., 2009; Wenthold et al., 1996).

The abundance of AMPA receptors at the synapse can be modulated through posttranslational modifications. Phosphorylation of AMPA receptor subunits on serine is a reversible post-translational modification and regulatory mechanism of expression and function of the receptor, as well as a relevant component of AMPA receptor trafficking and regulation of activity-dependent plasticity (Lu and Roche, 2012; Song and Huganir, 2002). Different phosphorylation sites including GluA1 Serine 845 (Roche et al., 1996) and GluA1 Serine 831 (Diering et al., 2014; Mammen et al., 1997) have been particularly characterized. Phosphorylation of Serine 845 by Protein Kinase A (PKA) is involved in AMPA receptor trafficking to the synaptic membrane (Esteban, 2003; Lee et al., 2000; Oh et al., 2006). Serine 831 is a site phosphorylated by Protein kinase C (PKC) (Roche et al., 1996) and calcium/calmodulin-dependent protein kinase II (CaMKII) (Mammen et al., 1997), and is related to the receptor response in several systems including long-term potentiation (LTP), a cellular model of learning and memory (Barria et al., 1997; Benke et al., 1998). Therefore, multiple phosphorylations on GluA1 AMPA receptor subunit have been suggested to cooperatively participate in the induction and maintenance of synaptic plasticity.

Previous studies reported that phosphorylation of the GluA1 subunit of AMPA receptor is modulated in learning and memory: during LTP (Lee et al., 2003; Song and Huganir, 2002), memory consolidation process (Bevilaqua et al., 2005; Ferretti et al., 2014), and memory reconsolidation process. Reconsolidation induces increased phosphorylation of GluA1 at Serine 845 (Jarome et al., 2012; Monfils et al., 2009) and Serine 831 after memory retrieval (Fukushima et al., 2014) and also a change in surface expression of the AMPA receptor subunit (Jarome et al., 2012; Lopez et al., 2015; Rao-ruiz et al., 2011; Yu et al., 2013).

The immune system is an important modulator of learning, memory, and neural plasticity. During neuroinflammation, elevated brain proinflammatory cytokines (such as TNF-α, IL-6 and IL-1β) can mediate sickness behavior and impair cognitive processes (Dantzer et al., 2008). Memory impairment and increased cytokine expression are observed in humans with disorders such as depression (Young et al., 2014), neurodegenerative disorders like Alzheimer's disease (AD) and Parkinson's disease (Cacquevel et al., 2004; Decourt et al., 2017; Nagatsu and Sawada, 2005; Perry et al., 2003), and infectious diseases (Shapira-Lichter et al., 2008). To abrogate cognitive deficits associated with neuroinflammation it is necessary to understand how cytokines affect brain function at behavioral and cellular levels. Interleukin 1β (IL-1β) is one of the main pro-inflammatory cytokines involved in sickness behavior and memory impairment. Several groups have reported that interleukin IL-1β has an inhibitory effect on LTP (Lynch, 2015). This effect is concentration-dependent (Coogan et al., 1999; Cunningham et al., 1996; Hoshino et al., 2017; Ross et al., 2003). The negative effect of IL-1β on LTP was also described in vivo and thus, intracerebroventricular injection of IL-1β (3.5 ng/ml) inhibited LTP in urethane-anaesthetized rats (Kelly et al., 2001). More recently, LTP in different hippocampal regions in mouse brain slices was impaired by IL-1β at a dose of 1 ng/ml (Hoshino et al., 2017). Our previous results showed that intrahippocampal administration of IL-1β (5ng/0.25 μl per side) was able to impaired memory consolidation and reconsolidation (Gonzalez et al., 2009; Machado et al., 2010). Besides, other authors working on the central effect of IL-1β have also used the same (Pugh et al., 2001, 1999; Yirmiya et al., 2002) or similar doses (Barrientos et al., 2004, 2002; Pugh et al., 2001), showing impairment of contextual or spatial memory. Other reports also showed that central administration of IL-1β at similar doses significantly induced inflammatory-sickness responses (Song et al., 2003; Song and Horrobin, 2004). Previous studies by our group have demonstrated that intrahippocampal administration of IL-1β after reactivation of contextual fear memory impairs reconsolidation process (Machado et al., 2010; see also Supplementary Fig. 1C). Therefore, it is important to elucidate the mechanisms involved in IL-1β-induced impairment in memory reconsolidation. We previously demonstrated that IL-1β induced a significant decrease of glutamate release and calcium influx in synaptosomes after reactivation of contextual fear memory. These events were dependent on memory reactivation, as we have shown by comparing not reactivated with reactivated groups (Machado et al., 2015). The postsynaptic consequences of these effects were observed as a lowering of *p*-ERK and Zif268 levels (Machado et al., 2015), and could probably affect glutamatergic receptors as well. In a hippocampal neuron culture, IL-1β influences phosphorylation and surface expression of GluA1 AMPA receptor subunit (Lai et al., 2006). This evidence suggests that IL-1β administration could also affect AMPA receptor activity.

The neuroimmunomodulator α-melanocyte-stimulating hormone (α-MSH) is an endogenous neuropeptide with multiple effects on the central nervous system (CNS) including anti-inflammatory properties. α-MSH exerts its effects through five different G protein-coupled receptors, the melanocortin receptors, (MC1R, MC2R, MC3R, MC4R and MC5R). MC3 and MC4 receptors are predominantly expressed throughout the CNS including the hippocampus (Muceniece and Dambrova, 2010). α-MSH through activation of MC4R prevents the effect of IL-1β on consolidation (Gonzalez et al., 2009) and reconsolidation (Machado et al., 2010) of a contextual fear memory. Also, activation of postsynaptic MC4R in the hippocampus facilitates synaptic transmission and plasticity through enhancement of AMPA receptor-mediated neurotransmission (Shen et al., 2013). Thus, activation of MC4R increases the number of mature dendrite spines and enhances surface expression of AMPA receptor subunit GluA1 (Shen et al., 2013). Moreover, MC4R stimulates surface GluA1 trafficking through phosphorylation of GluA1 at Serine 845 in a cAMP/PKA-dependent manner.

Based on these observations we investigated whether IL-1β administration can affect GluA1 AMPA receptor subunit phosphorylation, surface expression, and total expression during reconsolidation of a contextual fear memory. Also, we studied the modulatory effect of α-MSH.

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 two or 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 (rt) of 22 ± 1 °C. Behavioral testing was performed during the light cycle between 9:00 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 (RHCD 674/09). The number of animals used as well as their suffering was kept to the minimum necessary for the purposes of this study.

2.2. Surgery and drug infusion procedures

Animals were anaesthetized with an intraperitoneal 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 anterior posterior: −3.2 mm; lateral: ± 2.0 mm; dorso ventral: −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 micro-infusion 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 according to manufacturer specifications. The solution of 0.01% bovine serum albumin did not interfere with memory processes and did not cause any disturbance in the tissue surrounding the injection. α-MSH were dissolved in sterile saline (0.9% sodium chloride) and stored in aliquots at −20 °C also according to manufacturer specifications. 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), and sterile saline (0.9% sodium chloride, 0.25 μl) was used for control injections. The dose of IL-1β was established according to previous results from our group and also from other publications in the field (Gonzalez et al., 2009; Machado et al., 2010; Pugh et al., 2001; Yirmiya et al., 2002). Regarding α-MSH dose, we also tested it previously in our laboratory (Machado et al., 2010).

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, a context, such as a conditioning chamber, is presented with an aversive unconditional stimulus (US), a footshock. The pairing of the CS and the US elicits conditioned fear responses including increased freezing behavior.

The conditioning chamber was made of white acrylic (44 × 22 × 20 cm) with a 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 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 with experimenters unaware of the treatment condition.

2.4.1. Training

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; 2.0 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.

2.4.2. Re-exposure session (reactivation)

Re-exposure session (Reactivation) 24 h after training, animals were re-exposed to the training context without shocks for 3 min. Since the animals had not yet received any treatment at that moment, there were no differences in freezing percentage between them (Supplementary Fig. 1B).

In order to perform the biochemical experiments detailed below, the animals received intrahippocampal infusion of the different treatments immediately after the reactivation session, were killed at different times depending on the experiment, and dorsal hippocampus was dissected as soon as possible. Whenever a double infusion was necessary, there was a 10 min difference between the two.

2.4.3. Test session

Test session was only performed as a control of conditioning and drug effect on behavior (Supplementary Fig. 1C). Animals were again placed in the conditioned context for a 5 min period without receiving shock, 24 h after reactivation. Memory was assessed and expressed as the percentage of time rats spent freezing. This behavior is commonly used as an index of fear in rats (Blanchard and Blanchard, 1989). An animal was considered to be freezing when it was crouching without movement of the head or body, except those parts associated with breathing. The total time spent freezing in each period was quantified (in seconds) and expressed as percentage of total time.

2.5. Biochemical determinations

2.5.1. Western blotting

Animals were killed by decapitation 0, 30 or 60 min after memory reactivation and after receiving the treatments, and samples were immediately used for *p*-Ser845 GluA1, *p*-Ser831 GluA1, and GluA1 determination. 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. Next, the dorsal hippocampus was dissected using the rat brain atlas (Paxinos and Watson, 2007) as a guide, and total proteins were extracted as follows. 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 phenylmetylsulfonyl fluoride) and phosphatase inhibitor (1 mM sodium

orthovanadate), and centrifuged at 10000×*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 (70 μg/lane) were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membrane (BioRad).

For detection of *p*-Ser845 GluA1, *p*-Ser831 GluA1, and GluA1, 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 polyclonal anti-phospho-AMPA Receptor GluA1 Serine 831 (*p*-Ser831 GluA1) (1:200 Cell Signaling Technology), rabbit polyclonal anti-phospho-AMPA receptor GluA1 Serine 845 (*p*-Ser845 GluA1) (1:300, Cell Signaling Technology), rabbit monoclonal anti-AMPA receptor GluA1 (GluA1) (1:200, Cell Signaling Technology), or mouse monoclonal anti-actin (1:2,000, Sigma-Aldrich, Inc.), respectively. Blots were then incubated with anti-rabbit IRDye800CW or anti-mouse IRDye680 (1/25 000, Li-Cor) for 120 min at rt. Membranes were scanned on a Li-Cor Odyssey imager under non-saturating conditions. Data were processed on Odyssey software according to manufacturer protocols (Li-Cor, Nebraska, USA), and analyzed with an image analysis program (Gel-Pro Analyzer Software). Data are presented as the ratio of *p*-Ser831 GluA1/actin, *p*-Ser845 GluA1/actin and GluA1/actin in each sample.

2.5.2. Surface biotinylation

Animals were killed by decapitation 60 min after reactivation for AMPA receptor subunit GluA1 surface expression (GluA1-s) determination. Brains were quickly removed and dorsal hippocampus was dissected as described above for Western blot. The dissected area was transferred to ice-cold sulfo-NHS- LC-biotin (Pierce, Rockford, IL, USA) in phosphate-buffered saline (0.3 mg/mL) and incubated for 1 h, then rinsed in cold Tris-glycine to quench free biotin (5 min) followed by washes with ice-cold Tris-buffered saline (TBS; 3 × 5 min). Microdissected hippocampus was homogenized in 300 μL of RIPA buffer. Homogenates were centrifuged at 13 000 g for 30 min to pellet the insoluble fraction. For the total fraction of GluA1 (surface plus internal), 50 μL of the supernatant was mixed and heated with 4X SDS sample buffer. The remnant supernatant (200 μl) containing the biotinylated surface proteins was immunoprecipitated with 50 μL of 50% avidin-agarose beads (ImmunoPure Immobilized Avidin; Pierce) for 2 h at 4 °C. The beads were pelleted, and 150 μL of the supernatant (internal fraction) was mixed and heated with 4X SDS sample buffer. The beads were then rinsed three times with ice-cold TBS and heated in 50 μL of 2X SDS sample buffer (surface fraction). The surface fraction and surface plus internal were subjected to quantitative immunoblotting for AMPAR using anti-GluA1 (Cell Signaling). In the GluA1 plasma membrane expression study, plasma membrane-associated AMPA receptor GluA1 subunit was defined as the surface fraction of SDS sample buffer eluent collected from avidin beads, normalized to actin (in the total fraction) in each sample (Esparza et al., 2012). Quantitative immunoblotting for GluA1-s was similar to the procedure describe for GluA1 in the previous section.

2.6. Statistics

All data are expressed as mean ± standard error (SEM) and were analyzed by one-way or two-way ANOVA as appropriate. All data presented were analyzed for normality (Wilk-Shapiro) and equality of variance (Levene's Test) at a 0.05 significance level. If any statistically significant difference was found, post hoc analysis was performed using Newman–Keuls test. In all tests, differences with p values < 0.05 were considered statistically significant.

3. Results

3.1. IL-1β reduced phosphorylation of AMPA receptor subunit GluA1 at Serine 831 and Serine 845 after fear memory reactivation

We first determined GluA1 AMPA receptor subunit phosphorylation at Serine 831 (*p*-Ser831 GluA1) induced by memory reactivation and the effect of IL-1β on this process. Animals were trained and re-exposed to the context for memory reactivation, received an intrahippocampal infusion of saline (SAL) or IL-1β after memory reactivation, and were killed immediately, 30 or 60 min afterwards. Results are expressed as the relation *p*-Ser831 GluA1/actin. We found an increase in GluA1 AMPA receptor subunit phosphorylation at Serine 831 in saline treated animals 60 min after memory reactivation (Fig. 1C).

Administration of IL-1β immediately after memory reactivation reduced GluA1 AMPA receptor subunit phosphorylation at Serine 831 60 min after memory retrieval (Fig. 1C). Two-way ANOVA indicated a significant effect of treatment [$F(1,25) = 6.86$; $p < 0.05$], time [$F(2,25) = 5.84$; $p < 0.01$], and also significant interaction between these two factors [$F(2,25) = 7.33$; $p < 0.005$]. The post hoc Newman Keuls test showed that the SAL 60 min group was significantly different from SAL 0 and 30 min and from IL-1β 60 min.

We also studied GluA1 AMPA receptor subunit phosphorylation at Serine 845 (*p*-Ser845 GluA1) after memory retrieval. Similar to *p*-Ser831 GluA1, phosphorylation of GluA1 subunit at Serine 845 increased 60 min after memory reactivation. This increment was not observed if animals received intra-hippocampal infusion of IL-1β (Fig. 1F). Two-way ANOVA showed a significant effect of treatment [$F(1,36) = 5.15$; $p < 0.05$], time [$F(2,36) = 6.03$; $p < 0,01$] and interaction between variables [$F(2,36) = 8.14$; $p < 0.001$]. Newman Keuls test indicated a significant difference between the SAL 60 min group and the other saline groups. Also, the IL-1β 60 min group was statistically different from the SAL 60 min group.

3.2. α-MSH prevented the effect of IL-1β on GluA1 AMPA receptor subunit phosphorylation at Serine 845 but not at Serine 831

We evaluated the effect of α-MSH on the decrease in *p*-Ser831 GluA1 AMPA receptor subunit produced by IL-1β 60 min after memory reactivation. Treatment with α-MSH did not prevent the decrease in GluA1 AMPA receptor subunit phosphorylation (Fig. 2C). Analysis of results by one way ANOVA revealed a significant effect of treatment [$F(3,23) = 4.63$; $p < 0.05$]. Post hoc analysis by Newman Keuls test indicated that the SAL + SAL group was significantly different from IL-1β+SAL and from IL-1β+α-MSH but not from SAL+α-MSH.

Similarly, we assessed the effect of α-MSH on the reduction in *p*-Ser845 GluA1 AMPA receptor subunit induced by IL-1β 60 min after memory reactivation. Treatment with α-MSH prevented this effect, and levels of GluA1 AMPA receptor subunit phosphorylation at Serine 845, 60 min after memory reactivation, were similar to SAL group (Fig. 2F). One-way ANOVA indicated a significant effect of treatment [$F(3,22) = 8.43$; $p < 0.001$], and Newman Keuls test showed that IL-1β significantly reduced *p*-Ser845 GluA1 compared to the other groups; there were no significant differences between groups treated with SAL + SAL, IL-1β+α-MSH and SAL+α-MSH.

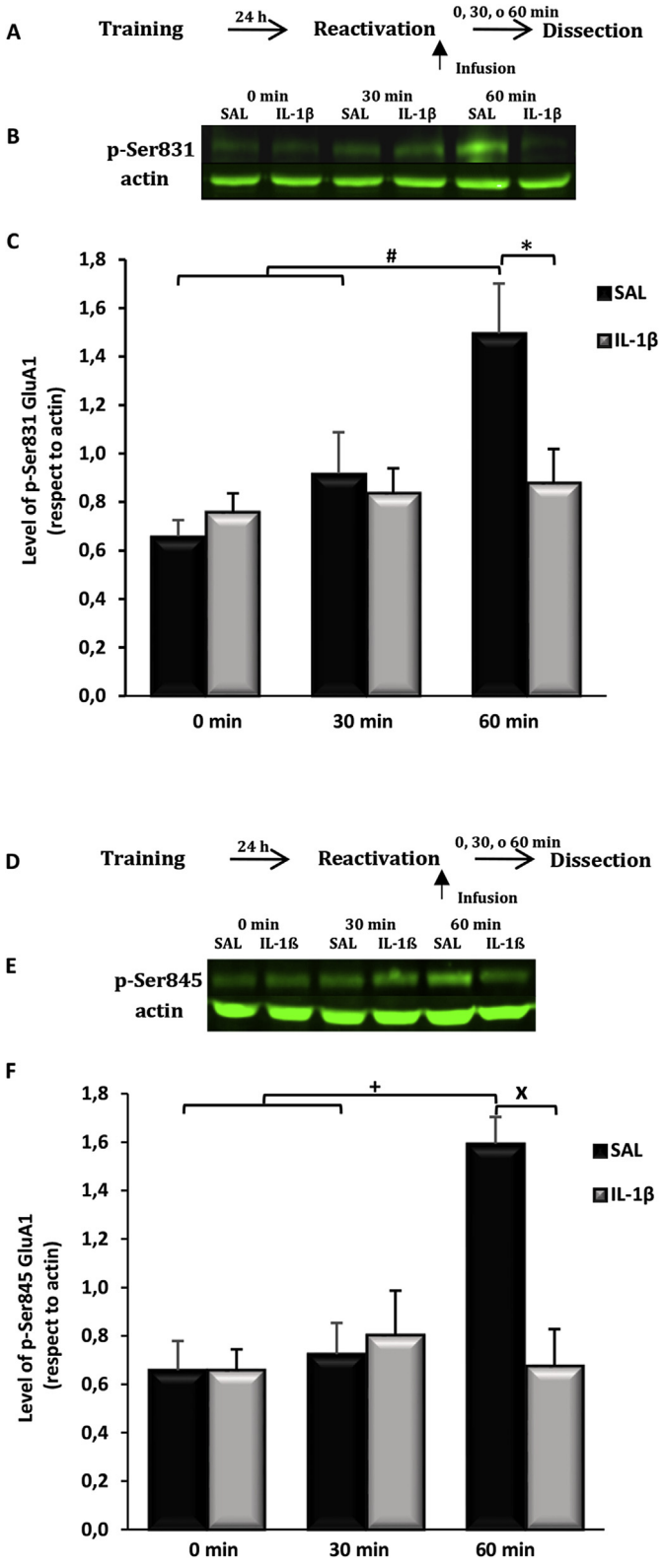

Fig. 1. IL-1β reduced phosphorylation of AMPA receptor subunit GluA1 at Serine 831 (*p*-Ser831 GluA1) and Serine 845 (*p*-Ser845 GluA1) after fear memory reactivation. Phosphorylation of GluA1 at Serine 831 and Serine 845 was analyzed by Western blot in total homogenates prepared from dorsal hippocampus. (**A and D**) Schematic representation of experimental procedures. (**B and E**) Representative Western blot of *p*-Ser831 GluA1, *p*-Ser845 GluA1 and actin levels. (**C and F**) Animals were injected immediately after retrieval of contextual fear memory with SAL or IL-1β and killed either immediately, 30 or 60 min later. Data are expressed as the mean of *p*-Ser831/actin or *p*-Ser845/actin ratio ± S.E.M. #$p < 0.01$ vs. SAL 0 and 30 min, *$p < 0.005$ vs SAL 60 min (**n = 4–7**); +$p < 0.001$ vs. SAL 0 and 30 min, **x** $p < 0.001$ vs SAL 60 min (**n = 4–9**).

3.3. IL-1β reduced AMPA receptor subunit GluA1 surface expression (GluA1-s) after memory reactivation and α-MSH prevented this effect

Phosphorylation of GluA1 AMPA receptor subunit is a regulatory mechanism of surface expression and function of the receptor. Considering that IL-1β reduced GluA1 AMPA receptor subunit phosphorylation, we next studied the effect of IL-1β on expression of this AMPA receptor subunit in plasma membrane. Different groups of animals received SAL + SAL, IL-1β+SAL, SAL+α-MSH or IL-1β+α-MSH after memory reactivation and hippocampus were removed 60 min afterwards (Fig. 3A). IL-1β decreased GluA1 AMPA receptor subunit surface expression and α-MSH reversed this effect (Fig. 3C). One way ANOVA showed a significant effect of treatment [$F(3,25) = 3.81$; $p < 0,001$]. Newman Keuls test indicated that the group treated with IL-1β+SAL was significantly different from the others. There was no statistical difference between the other groups.

3.4. IL-1β reduced the increase in AMPA receptor subunit GluA1 expression during memory reconsolidation and α-MSH prevented this effect

We also examined AMPA receptor subunit GluA1 expression in dorsal hippocampus in animals that received SAL + SAL, IL-1β+SAL, SAL+α-MSH or IL-1β+α-MSH after memory reactivation and were killed either immediately, 30 or 60 min later. We observed an increase in GluA1 expression 60 min after memory reactivation. This increment was not observed in the group treated with IL-1β (Fig. 4C). Two-way ANOVA indicated a significant effect of treatment [$F(1,34) = 6.81$; $p < 0.05$] and time [$F(2,34) = 4.18$; $p < 0.05$] as well as interaction between treatment and time [$F(2,34) = 5.62$; $p < 0.01$]. Post hoc analysis demonstrated a significant increment in GluA1 AMPA receptor subunit expression in the control group at 60 min compared to the other times studied and that expression of GluA1 AMPA receptor subunit was significantly different in animals that received IL-1β. If animals treated with IL-1β also received α-MSH, expression of GluA1 AMPA receptor subunit was similar to the control group (Fig. 4F). One way ANOVA showed a significant effect of treatment [$F(3,19) = 4.15$; $p < 0.05$]. Newman Keuls test indicated that expression of GluA1 in the group that received IL-1β was statistically different from the others at this particular time after memory reactivation.

4. Discussion

The molecular mechanisms involved in the effect of IL-1β on memory reconsolidation process could provide new insights into the participation of this cytokine in disorders characterized by elevated levels of cytokines (Cacquevel et al., 2004; Nagatsu and Sawada, 2005). Further, it has been proposed that memory reconsolidation serves to maintain, strengthen, or modify memories (Tronson and Taylor, 2007). Therefore, the study of reconsolidation memory impairment induced by IL-1β could contribute to understanding the mechanisms behind immune activation and memory updating. In order to identify cellular mechanisms induced by intrahippocampal administration of IL-1β that might lead to impairment of memory reconsolidation, we previously demonstrated that IL-1β reduces glutamate release during memory reconsolidation (Machado et al., 2015). Therefore, considering that AMPA receptors trafficking depends on glutamate (Derkach et al., 2007) IL-1β could also affect AMPA receptor phosphorylation and expression.

Consequently, we evaluated phosphorylation of GluA1 AMPA receptor subunit at Serine 845 and Serine 831 after memory

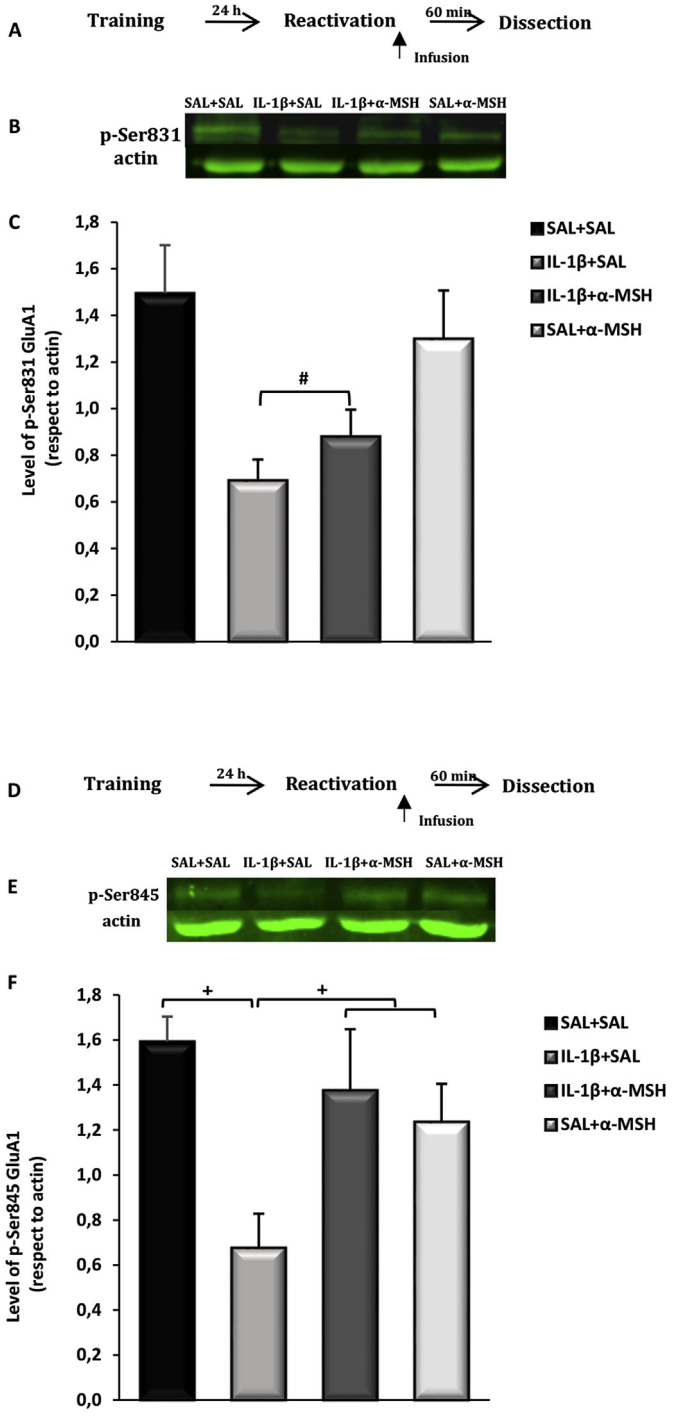

Fig. 2. α-MSH prevented the effect of IL-1β on GluA1 AMPA receptor subunit phosphorylation at Serine 845 but not at Serine 831. The phosphorylation of GluA1 at Serine 831 and Serine 845 was analyzed by Western blot in total homogenates prepared from dorsal hippocampus. (**A and D**) Schematic representation of experimental procedures. (**B and E**) Representative Western blot of *p*-Ser831 GluA1, *p*-Ser845 GluA1 and actin levels. (**C and F**) Animals were treated after reactivation of a contextual fear memory with SAL + SAL, IL-1β+SAL, IL-1β+α-MSH or SAL+α-MSH and were killed 60 min afterwards. Data are expressed as the mean of pSer831/actin or *p*-Ser845/actin ratio ± S.E.M. **#**$p < 0.05$ vs. other groups **(n = 5–8);** + $p < 0.05$ vs. other groups **(n = 5–8).**

reactivation and the effect of IL-1β on these processes. We observed an increase in GluA1 AMPA receptor subunit phosphorylation in both serines 60 min after reactivation of a contextual fear memory in hippocampus. Concordantly, it has been demonstrated that the reactivation of a fear memory associated with an auditory cue also induces an increase in *p*-Ser845 GluA1 AMPA receptor subunit, at the same time, in amygdala (Jarome et al., 2012). Other authors described an increment in *p*-Ser845 GluA1 AMPA receptor subunit 3 min and 60 min after reactivation of contextual fear memory, also in amygdala (Monfils et al., 2009) and in both serines 30 min after reactivation of an inhibitory avoidance memory in hippocampus, amygdala and cortex (Fukushima et al., 2014). These results suggest that GluA1 AMPA receptor subunit phosphorylation is an important event in memory processes, more specifically in memory reconsolidation.

We next evaluated the effect of IL-1β on phosphorylation of GluA1 AMPA receptor subunit during memory reconsolidation. IL-1β reduced phosphorylation of this subunit at Serine 831 and Serine 845, 60 min after contextual fear memory reactivation. However, we cannot exclude that IL-1β only delayed the increase in GluA1 AMPA receptor subunit phosphorylation after reactivation of memory.

GluA1 AMPA receptor subunit phosphorylation is associated with AMPA insertion at postsynaptic level (Esteban, 2003; Malinow, 2003; Shepherd and Huganir, 2007). Therefore, we tested whether the decrease in GluA1 AMPA receptor subunit phosphorylation induced by IL-1β could influence its surface expression. Intrahippocampal administration of IL-1β after memory reactivation induced a decrease in surface expression of GluA1 AMPA receptor subunit. Concordant with these results, IL-1β was shown to affect phosphorylation and also surface expression of AMPA receptors in hippocampal neurons in culture (Lai et al., 2006).

Signaling mechanisms controlling AMPA receptor trafficking are not yet totally understood. However, involvement of phosphorylated protein kinases such as ERK and p38 in synaptic delivery of AMPA receptor has been demonstrated (Corrêa and Eales, 2012; Esteban, 2003; Seger and Krebs, 1995; Zhu et al., 2002). ERK phosphorylation induces surface expression of AMPA receptor during LTP by a mechanism mediated by the small GTPase, Ras (Esteban, 2003; Zhu et al., 2002). We demonstrated that IL-1β administration reduced ERK phosphorylation during memory reconsolidation (Machado et al., 2015). Also, p38 activation mediates AMPA receptor endocytosis through a mechanism involving another small GTPase, Rap (Zhu et al., 2002; Esteban, 2003). Activation of IL-1 receptor type I (IL-1RI) by IL-1β increased p38 phosphorylation (Srinivasan et al., 2004). Previous results from our lab demonstrated that intrahippocampal administration of IL-1β increases p38 phosphorylation and that treatment with a p38 inhibitor partially reverses IL-1β effect on contextual fear memory consolidation (Gonzalez et al., 2013). Therefore, IL-1β could affect GluA1 surface expression by decreasing ERK2 phosphorylation and/or increasing p38 activation. Further studies will be needed to confirm this hypothesis and elucidate mechanisms involved.

We determined the effect of α-MSH on the decrease in GluA1 AMPA receptor subunit phosphorylation induced by IL-1β during memory reconsolidation. α-MSH prevented the effect of IL-1β on GluA1 AMPA receptor subunit phosphorylation in Serine 845, but not in Serine 831. Further, treatment with α-MSH also prevented the effect of this cytokine on GluA1 AMPA receptor subunit surface expression after memory reactivation. MC4R activation is associated with the G protein-AMPc-PKA pathway and PKA is involved in GluA1 phosphorylation at Serine 845 (Abel and Nguyen, 2008). Concordantly, MC4R activation stimulates GluA1 AMPA receptor subunit phosphorylation at Serine 845 and surface expression of this subunit in hippocampal neurons in culture without affecting phosphorylation at Serine 831 (Shen et al., 2013). These results agree with ours; thus, the effect of α-MSH on Serine 845 GluA1

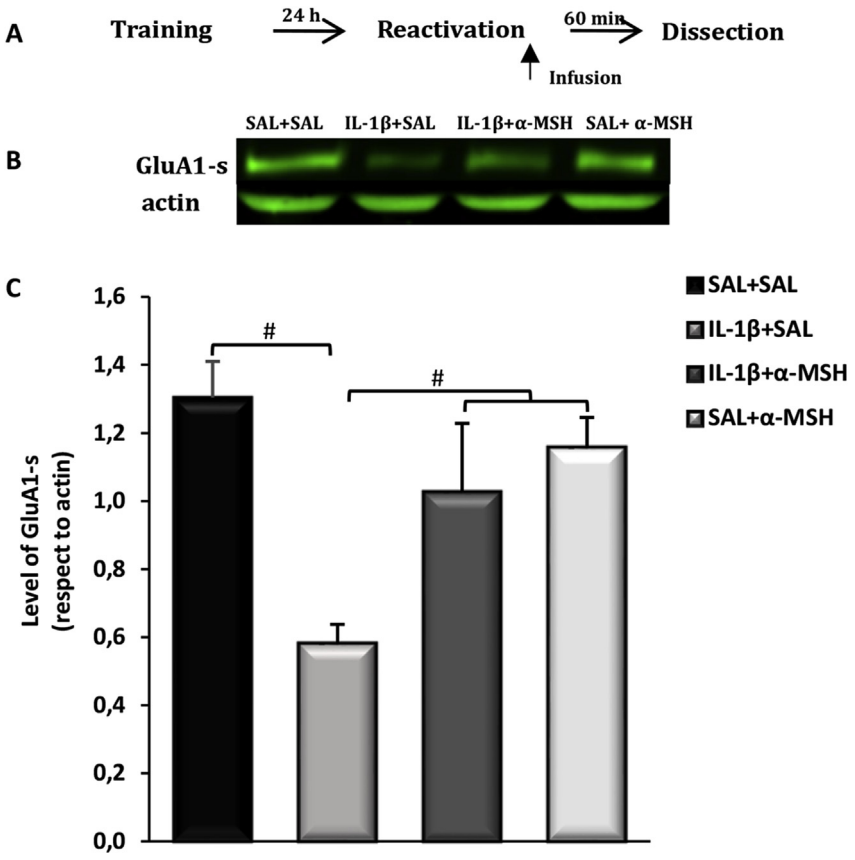

Fig. 3. IL-1β reduced AMPA receptor subunit GluA1 surface expression (GluA1-s) after memory reactivation and α-MSH prevented this effect. The level of GluA1-s was analyzed by Western blot of biotinylated surface proteins from dorsal hippocampus. **(A)** Schematic representation of experimental procedures. **(B)** Representative Western blot of GluA1-s and actin levels. **(C)** Animals were treated after reactivation of a contextual fear memory with SAL + SAL, IL-1β+SAL, IL-1β+α-MSH or SAL+α-MSH and were killed 60 min afterwards. Data are expressed as the mean of GluA1-s/actin ratio ± S.E.M. #p < 0.05 vs other groups **(n = 5–8).**

AMPA receptor subunit phosphorylation and the membrane insertion of this subunit could be related to MC4R activation at postsynaptic level. Phosphorylation of Serine 845 GluA1 AMPA receptor subunit and CaMKII activation, but not GluA1 AMPA receptor subunit phosphorylation at Serine 831, have been shown to be necessary for surface expression of AMPA receptors containing this subunit (Esteban et al., 2003; Hayashi et al., 2000). This evidence supports our results: α-MSH can reverse the decrease of GluA1 AMPA receptor subunit surface expression induced by IL-1β, although it did not reverse the decline in Serine 831 GluA1 AMPA receptor subunit phosphorylation. Numerous studies have indicated that maintaining a fear memory after retrieval requires de novo protein synthesis. However, no study to date has examined how GluA1 level expression changes over time after retrieval. The present study determined the expression of AMPA receptor subunit GluA1 at different times after memory reactivation. We observed that expression of GluA1 AMPA receptor subunit increased 60 min after memory reactivation in hippocampus. To our knowledge, this result is the first evidence of changes in GluA1 expression during memory reconsolidation. However, up-regulation of GluA1 subunit has been described during extinction of cocaine-seeking (Sutton et al., 2003) and in several paradigms of drug reward (Carlezon et al., 1997; Carlezon and Nestler, 2002; Glass et al., 2005; Olson et al., 2005). In this study, we also found that IL-1β administration impeded the increase in expression of GluA1 and α-MSH prevented this effect.

Our results demonstrate that IL-1β regulates phosphorylation of two distinct sites on GluA1 AMPA receptor subunit and so, may play an important role in the modulation of AMPA receptor function and synaptic plasticity in the brain. Impairments in processes that control AMPA receptor trafficking or activity can have direct consequences on brain function. Modulation of calcium-permeable AMPA receptors and consequently a reduction in synaptic transmission in the lateral amygdala has also been associated with memory disruption in a reconsolidation extinction interference protocol (Clem and Huganir, 2010). Also, disruption of AMPA trafficking during LTP and LTD by soluble amyloid-β oligomers is one of the most important factors of synaptic dysfunction in AD (Henley and Wilkinson, 2016).

We previously observed that IL-1β treatment impairs reconsolidation of a contextual fear memory, possibly through a presynaptic mechanism involving Ca2+ influx and glutamate release in dorsal hippocampus (Machado et al., 2015). GluA1 AMPA receptor subunit phosphorylation, surface expression, and total expression are postsynaptic events dependent on glutamate and memory reactivation (Derkach et al., 2007; Monfils et al., 2009). Therefore, a decrease in glutamate release after memory reactivation induced by IL-1β (Machado et al., 2015) could be related to the observed reduction in AMPA receptor subunit GluA1 phosphorylation, surface expression, and total expression. Furthermore, the biochemical changes we reported are supported by behavioral consequences, i.e. a decrease in memory reconsolidation.

Our findings further illustrate the importance of IL-1β in cognition processes that depend on hippocampus and also reinforce the fact that α-MSH can reverse IL-1β effects on memory reconsolidation. In conclusion, our novel findings provide another

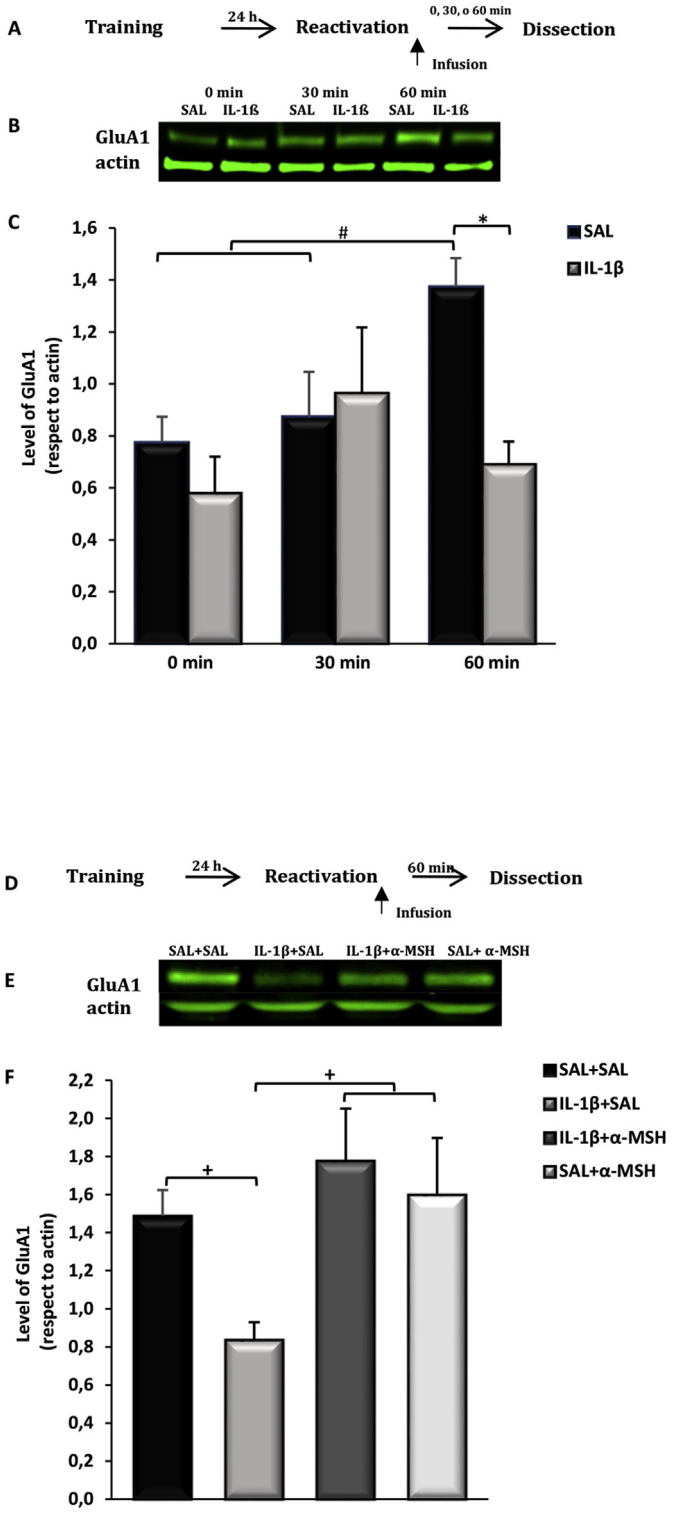

Fig. 4. IL-1β reduced the increase in AMPA receptor subunit GluA1 expression after memory reactivation; α-MSH prevented this effect. Levels of GluA1 were analyzed by Western blot in total homogenates prepared from dorsal hippocampus. (**A and D**) Schematic representation of experimental procedures. (**B and E**) Representative Western blot of GluA1 and actin levels. (**C**) Animals were injected immediately after retrieval of a contextual fear memory with SAL or IL-1β and killed immediately, 30, or 60 min afterwards. (**F**) Animals were treated after reactivation of contextual fear memory with SAL + SAL, IL-1β+SAL, IL-1β+α-MSH or SAL+α-MSH and killed 60 min later. Data are expressed as the mean of GluA1/actin ratio ± S.E.M. #$p < 0.05$ vs. SAL 0 and 30 min, *$p < 0.05$ vs SAL 60 min ($n = 5–9$); +$p < 0.05$ vs other groups (**n = 5–7**).

plausible molecular mechanism underlying the inability to perform memory-related tasks under immune stress, and the importance of α-MSH as neuromodulator.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2017.09.041.

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