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ORIGINAL INVESTIGATION

Ghrelin increases memory consolidation through hippocampal mechanisms dependent on glutamate release and NR2B-subunits of the NMDA receptor

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Abstract
Rationale Ghrelin (Ghr) is a peptide that participates in the modulation of several biological processes. Ghr administration into the hippocampus improves learning and memory in different memory tests. However, the possible mechanisms underlying this effect on memory have not yet been clarified.
Objective The purpose of the present work is to add new insights about the mechanisms by which Ghr modulates long-term memory consolidation in the hippocampus. We examined Ghr effects upon processes related to increased synaptic efficacy as presynaptic glutamate release and changes in the expression of the NR2B-subunits containing *n*-methyl-d-aspartate receptors (NMDAR), which are critical for LTP induction. We also attempted to determine the temporal window in which Ghr administration induces memory facilitation and if the described effects depend on GHS-R1a stimulation.
Results The present research demonstrated that Ghr increased glutamate release from hippocampal synaptosomes; intrahippocampal Ghr administration increased NR2B-subunits expression in CA1 and DG subareas and also reversed the deleterious effects of the NR2B-subunit-specific antagonist, Ro 25-6981, upon memory consolidation and LTP generation in the hippocampus. These effects are likely to be the consequence of GHS-R1a activation.
Conclusion According to the results above mentioned and previous findings, we can hypothesize some of the mechanisms by which Ghr modulates memory consolidation. At presynaptic level, Ghr stimulates glutamate release, probably by enhancing $[Ca^{2+}]_i$. At postsynaptic level, the glutamate released activates NMDAR while Ghr also mediates effects directly activating its specific receptors and increases NR2B-subunit expression.

Keywords Ghrelin · Memory consolidation · Glutamate release · LTP · NR2B subunit

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Introduction

The limitations of current pharmacological treatments of neurodegenerative diseases such as Alzheimer and Parkinson have led to extensive research about novel drugs that delay the progression or provide symptoms relief (Dos Santos et al. 2013). In this context, the knowledge about neurobiological bases of ghrelin (Ghr) effects in the central nervous system could contribute to highlight recent findings showing its potential as a new palliative agent in neurodegenerative diseases.

Ghr is a 28-amino acid peptide that participates in the modulation of several processes related to energy homeostasis, gastrointestinal functions, anxiety-like behavior, and growth hormone-releasing activity (Kojima and Kangawa

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2005; Lago et al. 2005). This peptide is an endogenous ligand for the growth hormone secretagogue receptors (GHS-R), (Howard et al. 1996; Bednarek et al. 2000; Smith et al. 2001). The Ghr receptor GHS-R1a type is expressed in the central nervous system mainly in the hypothalamus, and it mediates the Ghr orexigenic effects (Tschop et al. 2000; Nakazato et al. 2001). However, it is also expressed in extra-hypothalamic structures such as the hippocampus (hp), a brain structure related to learning and memory. The hp is one of the few brain regions that express high levels of GHS-R1a (Bennett et al. 1997; Guan et al. 1997; Lattuada et al. 2013).

In a previous work, we have shown that Ghr administration in rats, either intra-cerebroventricularly or directly into brain areas such as the hp, amygdala, or dorsal raphe nucleus, enhances memory consolidation in tests such as step down (SDT) and object recognition in a dose-dependent manner (Carlini et al. 2002, 2004, 2008). In electrophysiological studies, we have also demonstrated that intra-hippocampal Ghr administration increases the hp excitability facilitating induction of long-term potentiation (LTP) (Carlini et al. 2010). Nevertheless, the molecular and cellular bases of Ghr effects in memory processes and how the peptide may alter synaptic plasticity and cognition remain still unclear and require further study.

LTP is considered to be a model of neural changes underlying learning and memory formation. The main events during memory consolidation coincide with the signaling pathways activated during the LTP induction in the hippocampal CA1 and dentate gyrus (DG) (Izquierdo et al. 2006). The biochemical memory cascade and LTP in those hippocampal regions are initiated by presynaptic glutamate (Glu) release and activation of ionotropic Glu receptors at the postsynaptic membrane such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and *n*-methyl-d-aspartate receptors (NMDAR). NMDAR activation induces inflow of Ca^{+2} through the receptor-associated ion channel, increasing intra-cellular calcium levels ($[Ca^{2+}]_i$) and activating different enzymes such as nitric oxide synthase (NOS) and several protein kinases (Izquierdo and Medina 1997). Thus, in the present work, we analyzed, among other issues, Glu release from hippocampal synaptosomes after Ghr administration in order to elucidate if Ghr effects upon memory could be related to enhancement of Glu release.

It has been demonstrated that both NR2A and NR2B subunits from NMDAR play important roles in both LTP and associative learning (Sakimura et al. 1995; Valenzuela-Harrington et al. 2007). The NMDAR is a main target for the development of cognitive enhancers because of its fundamental role in learning and memory. It has been suggested that LTP induction critically requires NR2B-subunits containing NMDAR (Barria and Malinow 2005). The NR2B-subunits improve synaptic plasticity and memory when over-expressed in mouse, leading to a larger hippocampal LTP and enhanced learning and memory function as tested in different memory tasks (Tang et al. 1999, 2001; Cao et al. 2007; Jacobs and Tsien 2012; Cui et al. 2011). On the contrary, genetic deletion of NR2B- subunits in the forebrain or hp (specific NR2B-subunit knockout animals) results in profound memory deficits and impaired LTP (Sprengel et al. 1998; von Engelhardt et al. 2008). In addition, a re-arrangement of the NMDAR subunit composition under different situations has also been observed; for instance in aging, a switch from NR2B to NR2A in NMDAR has been observed (Monyer et al. 1994; Sheng et al. 1994). Thus, differential assembly of the subunits of the NMDAR at the postsynaptic membrane, results in channels with different functional properties. In addition, NR2B-containing NMDAR promotes activation of neuronal NOS (nNOS) and nitric oxide (NO) synthesis (Brenman et al. 1996; Bredt et al. 1990; Vincent and Kimura 1992). NO modulates neuronal excitability and synaptic plasticity in different brain structures including the hp (Prast and Philippu 2001; Bartus et al. 2013).

In order to add new insights about postsynaptic mechanisms by which the peptide improves synaptic plasticity and memory, we explored the hypothesis that changes in functional properties in hp could be a consequence of increased NR2B-subunit expression. In addition, we studied the temporal window for Ghr-induced memory facilitation when it was administered posttraining and the participation of GHS-R1a in these effects by using a selective antagonist prior to Ghr administration.

In summary, we combined behavioral paradigms, electrophysiology, and molecular biology techniques in order to find out if the increase in synaptic efficacy induced by the peptide in hp could be related to: changes in Glu release from synaptosomes and variations in the expression of the NR2B-subunits containing NMDAR. We also studied the temporal window in which Ghr administration induces memory facilitation and if the described effects depend on specific stimulation of GHS-R1a.

Materials and methods

Animals

Adult male Wistar rats, weighing 280–300 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. Food and water were available ad libitum. Animals were kept on a 12-h light/dark cycle (lights on 7:00–19:00 hours) with a constant room temperature of 22±1 °C. The experiments were performed in accordance to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals and 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 were kept to the minimum in order to accomplish the goals of this study.

Surgery

Rats were anesthetized with intra-peritoneal administration of ketamine hydrochloride (55 mg/kg) and xylazine (11 mg/kg) and implanted bilaterally, with 22-gauge guide cannulae in the dorsal CA1 region of the hippocampus (coordinates A, −3.3 mm; L, ±2.0 mm; V, −2.0 mm) according to the atlas of Paxinos and Watson (2009). The cannulae were fixed to the skull with a screw and dental acrylic.

Drug infusion procedures

After surgery, animals were handled gently every day and habituated to intra-hippocampal injections throughout the recovery period. The behavioral tests started 7 days after surgery. To perform local infusions into the hippocampus, rats were hand restrained and drug solutions or vehicle were administered with infusion cannulae (30 gauge). Infusion cannula fitted into and extended 1 mm beyond the guide cannula. The infusion cannulae were connected, via polyethylene tubing (PE 10; Becton Dickinson, Sparks, MD), to 10-μl micro-syringes (Hamilton, Reno, NV) mounted on a micro-infusion pump (Harvard Apparatus, Holliston, MA). Each rat was injected with 0.5 μl/side at 0.5 μl/min flow rate. To allow drug diffusion, infusion cannulae were kept in place for a minute after infusion. Ghr concentration in infusion solution was 3 nmol/μl, according to previous experiments (see Carlini et al. 2002, 2004, 2010). Ghr (SC1356) was from polypeptide; Ro 25-6981 hydrochloride (R7150) and [D-Lys3]-growth hormone releasing peptide 6 (D-Lys^3-GHRP-6) (G4535) from Sigma-Aldrich.

Histological procedures

After behavioral tests, rats were killed by an overdose of chloral hydrate at 16%; their brains were removed and immersion fixed in a 4% formalin solution. Frontal sections were cut in a cryostat (Leica, Nussloch, Germany), the injection sites localized, and the extent of tissue damage caused by cannulation was examined under a light microscope. The injection sites were drawn on plates taken from a rat brain atlas (Paxinos and Watson 2009). Only data obtained from the animals with correct cannulae placement were included in the study.

Step-down test (inhibitory avoidance)

SDT has been widely used for the study of memory consolidation. One-trial step down has long been a favorite model for biochemical and pharmacological studies of memory. SDT learning relies on the formation of an association between stepping down from a platform with an aversive stimulus as foot-shock establishing a long-term memory expressed as an increase in step-down latency at testing.

Rats were subjected to one trial in the SDT. The training apparatus was a 50×25×25 cm plastic box with 2.5 cm high and 7.0 cm wide platform on the left of the training box apparatus. The floor of the apparatus was made of parallel 0.1 cm diameter stainless steel bars spaced 1.0 cm apart from each other. The animals were placed on the platform, and latency time to step down was measured (s) by placing the four paws on the grid.

In the training session, immediately upon stepping down, rats received a 0.4-mA, 2-s scrambled shock to the feet, and were then immediately removed from the training box and placed in their home cages. A retention test was carried out 24 h after training in order to measure long-term memory. Test sessions were identical to the training session except for the fact that no shock was given. A ceiling of 180 s was imposed on the retention test measures. An increase in the latency to step down was taken as measure of memory retention. Since the variables of the SDT did not follow a normal distribution, they were expressed as medians (inter-quartile range) and analyzed by non-parametric tests (the Scheirer–Ray–Hare extension of the Kruskal–Wallis test). The Scheirer–Ray–Hare test was performed on Predictive Analytical Software (version 18, SPSS Inc, Chicago, IL, USA) with additional calculations and a Chi-square table for determining the p value. Given the reduced power of the S-R-H test, a more conservative significance level was set at α=0.01 for these data.

Electrophysiological procedures

In order to evaluate if the changes on memory retention induced by intra-hippocampal Ghr administration could be correlated with the changes in the hippocampal dentate gyrus excitability, some animals were killed for the electrophysiological experiments immediately after the test session (24 h after training and Ghr administration) in the SDT. To prevent variations caused by circadian rhythms or non-specific stressors, rats were killed between 11:00 a.m. and noon (Teyler and Di Scenna 1987). The hippocampal formation was dissected and transverse slices of approximately 400-μm thick were maintained in a storage chamber containing standard Krebs solution (NaCl, 124.3 mM; KCl, 4.9 mM; $MgSO_4 \cdot 7H_2O$, 1.3 mM; H_2KPO_4, 1.25 mM; $HNaCO_3$, 25.6 mM; glucose, 10.4 mM; $CaCl_2 \cdot 2H_2O$, 2.3 mM; Sigma, Argentina) saturated with 95% O_2 and 5% CO_2. At the beginning of the experiments, a single slice was placed in a recording chamber (BSC-BU Harvard Apparatus) perfused with the standard Krebs solution saturated with 95% O_2 and 5% CO_2. The perfusion rate was 1.6 ml/min, and the bathing solution temperature was kept at 28 °C with a Temperature Controller (TC-202A Harvard Aparatus). A stimulating electrode made

of two twisted wires, which were insulated except for the cut ends (diameter 50 μm), was placed in the perforant path (PP). Then, a recording microelectrode was inserted in the dentate granule cell body layer. Only slices showing a stable response were included in the study. Amplitude (mV) of field excitatory postsynaptic potentials (EPSP) that responded to 0.2-Hz stimuli were sampled for 40 min until EPSP stabilization (baseline). Once no further changes were observed in the amplitude of EPSP, one of the two stimulation protocols were applied (Perez et al. 2010). Both protocols were tested in each animal.

In the first protocol, we assayed different stimulating frequency values in order to determine the minimum value to generate LTP (we call this value "threshold"). The stimulus consisted in a train of square pulses of 2-s length, with 0.5 ms being the duration of each square pulse. We used a stimulus frequency ranging from 5 to 200 Hz, delivered by an A310 accupulser pulse generator (World Precision Instruments Inc.). LTP was considered to have occurred when the EPSP amplitude recorded after the stimulus had risen at least 30% from baseline and persisted for 60 min. If LTP was not observed 20 min after of a given stimulation frequency, another hippocampal slice was used to test a stimulus at the next frequency value. Results were expressed as threshold mean (Hz)±standard error (SE) and analyzed by one-way analysis of variance (ANOVA).

For the second protocol, LTP was generated using the classical tetanization paradigm consisting of three 100-Hz high-frequency stimulation (HFS) trains (of 1 s duration each) given at 20 s intervals. LTP was considered to have occurred as described above for the first protocol. Then, results are expressed as percent of EPSP amplitude change related to the baseline±SE and analyzed by repeated ANOVA measures (time). For both protocols analysis, significance level was set to α=0.05, and the post hoc Student–Newman–Keuls (SNK) test was employed (Perez et al. 2010).

Study of glutamate release

Preparation of hippocampal synaptosomes

Rats were decapitated with a guillotine, and their brains were removed. Hp was dissected, and the synaptosomes were purified by using discontinuous Percoll gradients as previously described (Dunkley et al. 1988). Synaptosomes that sedimented between the 10 and 23% Percoll bands were collected and diluted in a final volume of 30 ml of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer medium (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). Samples were centrifuged at 27,000×*g* for 10 min at 4 °C. Pellets were re-suspended in 5 ml of HEPES buffer medium, stored in ice, and used within 3–4 h. Protein content was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA), and Glu release was performed after determination of total protein.

Glutamate release assay

Glu release from hippocampal synaptosomes was monitored online, using an assay employing exogenous Glu dehydrogenase and NADP+ to couple the oxidative decarboxylation of the released Glu. Then, the generated NADPH was detected fluorometrically (Nicholls et al. 1987; Vilcaes et al. 2009). Briefly, synaptosomal pellets were re-suspended in HEPES buffer medium (HBM) and incubated in a stirred and thermostated cuvette maintained at 37 °C in a FluoroMax-P Horiba Jobin Yvon Spectrofluorimeter (Horiba Ltd, Kyoto, Japan); 1 mM NADP+, 50 units/ml Glu dehydrogenase, and 1.2 mM $CaCl_2$ were added after 3 min. After 5 min of incubation, 3 mM 4-aminopyridine (4AP) was added to stimulate the Glu release. The synaptosomes were incubated in the presence of Ghr or HBM (control) 2 min prior to the addition of 4AP. A standard of exogenous Glu (4 nmol) was added at the end of each experiment, and the fluorescence response used to calculate Glu release that was expressed as nanomoles Glu per milligram of synaptosomal protein (nmol/mg) as well as percent of the total evoked-glutamate release in the control, considering the control as 100%. Data points were obtained at 1-s intervals. Quantification of Glu release was made by using the values obtained at 10 min of the experimental period and expressed as mean±SE, and also by calculating the area under the curve (AUC) using the "Origin8" software in order to analyze the total Glu released during 10 min of the experiment. For both parameters, each value was obtained from the four independent experiments performed by triplicate. Results were analyzed using the one-way ANOVA. Whenever ANOVA indicated significant effects (p<0.05), a pair-wise comparison of means by SNK was carried out. In all cases, the assumptions of the analysis of variance (homogeneity of variance and normal distribution) were attained.

Glu dehydrogenase (EC 1.4.1.3), NADP+, and 4AP were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Percoll was obtained from Pharmacia (Peapack, NJ, USA). All other chemicals were of analytical grade.

Immunohistochemistry

Twenty-four hours after Ghr administration, immediately after posttraining, animals were anesthetized with chloral hydrate at 16% (400 mg/kg i.p.) and transcardially perfused with 250 ml of saline (NaCl at 0.9%) and heparine (200 μl/l), followed by 400 ml of paraformaldehyde (PFA) at 4% in 0.1 M phosphate buffer (PB; pH 7.4). Brains were removed and kept overnight in PFA and then stored at 4 °C in PB containing 30% sucrose. Coronal

sections of 40 μm were cut using a cryostat (Leica CM15105) and collected in phosphate-buffered saline (PBS) at 0.01 M. They were placed in a mixture of 10% H_2O_2 and 10% methanol until oxygen bubbles ceased appearing. Samples were incubated in a mixture of 10% normal horse serum (NHS; GIBCO, Auckland, NZ), 0.1 % Triton X-100 (Fluka Analytical) in PBS at 0.01 M and 1 % bovine serum albumin (BSA; FEDESA S.A) for 2 h to block non-specific binding sites. The free-floating sections were incubated for 48 h at 4°C with a rabbit anti-NMDAR2B polyclonal antibody (AB1557P, Millipore), diluted 1:500 in PBS containing 1% NHS, 1% BSA, and 0.1 % Triton X-100. The sections were then rinsed with PBS at 0.01 M and incubated with biotin-labeled universal secondary antibody (diluted 1:1000 in 1% NHS-PBS) and avidin-biotin-peroxidase complex (diluted 1:200 in 1% NHS-PBS; Vector Laboratories, Burlingame, CA) for 2 h each at room temperature. The peroxidase label was detected with diaminobenzidine hydrochloride (Sigma Chemical Co.); the solution was intensified with 1% cobalt chloride and 1% nickel ammonium sulfate. This method produces a violet nuclear reaction product. Finally, the free-floating sections were mounted on gelatinized slides, air-dried overnight, dehydrated, cleared in xylene, and placed under a coverslip with DPX mountant for histology (Fluka Analytical). DPX Mountant for histology is a mixture of distyrene, a plasticizer, and xylene used as a synthetic resin-mounting media that replaces xylene-balsam. DPX Mountant dries quickly and preserves staining.

Quantification of NR2B-labeled cells

Images containing NR2B-immunoreactive cells (NR2B-IR) were obtained by using a computerized system that included a Leica DM 4000 B microscope equipped with a DFC Leica digital camera attached to a contrast enhancement device. The NR2B-IR cells were identified within the hippocampus at the dorsal region of CA1 and DG (corresponding to plates with a distance of −3.14 to −3.60 mm from Bregma) according to the atlas of Paxinos and Watson (2009). In the CA1, only pyramidal cells were quantified; in DG, only polymorphic cells were counted. Bilateral images for each hippocampal area were captured, in a total of four per animal concurrently for subjects across all groups. Counting of NR2B-IR was accomplished using IMAGE J software from the National Institutes of Health (NIH), considering a 0.09-mm^2 area (corresponding to ×400 magnification). The value obtained per animal was the average of positive cells in the four images. Counting of NR2B-IR cells was performed blinded to the experimental groups. The results were expressed as the mean±SE. The NR2B-IR were analyzed using one-way ANOVA. Whenever ANOVA indicated significant effects ($p<0.05$), a post hoc SNK test was employed.

Results

Ghrelin effect on memory consolidation studied in SDT

A) Temporal specificity of Ghr administration

In SDT paradigm, the animals learn (during the training) that stepping down from a platform is followed by a foot-shock; on subsequent exposure to the task (testing), they will stay much longer on the safe platform before eventually stepping down. Consequently, in this task an increase in latency time to step down evaluated 24 h after, indicating that the animal was able to learn and to remember the aversive stimulus (electrical shock), suggesting an increase in memory retention (long-term memory).

In a previous work, we have demonstrated that Ghr increases memory retention when it was intrahippocampally injected immediately after training and evaluated 24 h after, for long-term memory in the SDT (Carlini et al. 2002, 2004). Then, it was reasonable to inquire if there is a temporal window for Ghr effects on memory consolidation. This knowledge would allow us to suggest the step of memory cascade in which Ghr exert its actions. In order to answer this question, the experiments with Ghr administered immediately after training session (0 min) were repeated and compared with those obtained when Ghr or saline were administrated 15 or 60 min.after the training session in SDT (Fig. 1). As it was previously described, we demonstrated that animals which received Ghr immediately after training showed a significant increase in the latency time when compared with those from the control group (saline) ($F_{(2, 31)}=62.3$; $p<0.01$). Nevertheless, no differences in latency time were observed between groups administered 15 or 60 min when compared with the control group, indicating that Ghr does not modify the memory consolidation in the SDT when it was administered at these times.

B) Dependence on GHS-R1a

In order to determine if the behavioral and electrophysiological effects previously observed were mediated by direct activation of hippocampal GHS-R1a, the selective GHS-R1a antagonist, D-Lys^3-GHRP-6 or saline were infused before Ghr administration at 0 time after training session in SDT. Memory retention and electrophysiological parameters were studied 24 h after training session (Fig. 2). In the behavioral paradigm, a significant increase in latency time was observed in saline+Ghr group compared with saline+saline. When the antagonist D-Lys^3-

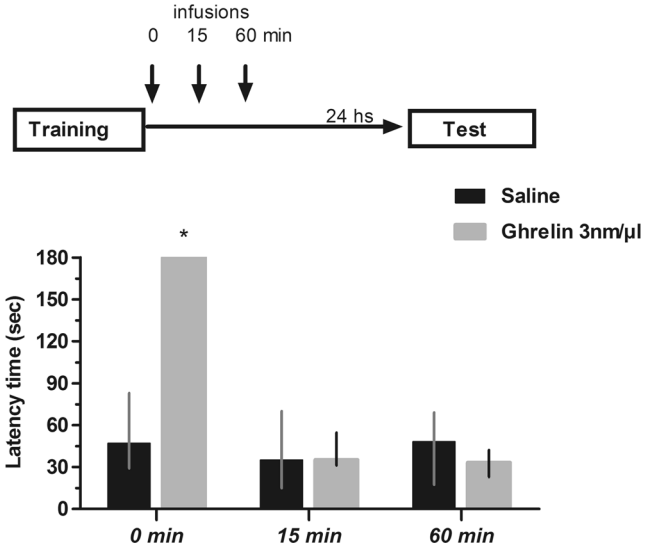


Fig. 1 Temporal specificity of Ghr administration on memory consolidation evaluated in SDT. The peptide (3.0 nmol/µl) was injected immediately (0), 15, and 60 min posttraining, and the latency time was measured 24 h later for long-term memory. The animals were injected with saline or with Ghr 3.0 nmol/µl. The results are expressed as medians of latency time with the respective inter-quartile range. N=8–10 animals/group. *$p<0.01$, significant differences related to the control animals (saline)

GHRP-6 was administered prior to Ghr (D-Lys^3-GHRP-6+Ghr group), no differences were observed in latency time compared with saline+saline. The administration of the antagonist alone (D-Lys^3-GHRP-6+saline group) did not modify the animals performance in relation to the saline+saline group (Fig. 2a). Statistical analysis of step-down latencies during testing (non-parametric two-way ANOVA) showed a significant saline or D-Lys^3-GHRP-6 vs. saline or Ghr interaction ($H_{(1)}$=9.1; $p<0.01$), revealing that co-injection of the selective antagonist prevented facilitation of memory induced by Ghr.

In order to determine if LTP facilitation induced by Ghr also depends on GHS-R1a activation, we tested differences in the degree of excitability in the slices from saline+saline, D-Lys^3-GHRP-6+saline, saline+Ghr, and D-Lys^3-GHRP-6+Ghr-treated groups analyzing the frequency values ("threshold") in hertz necessary to induce LTP in DG of hippocampal slices. As it can be seen in this set of experiments (Fig. 2d), and in accordance with our previous findings, a significant reduction in the threshold (Hz) to generate LTP was observed in slices from saline+Ghr-infused animals compared with those infused with saline+saline (saline+Ghr=7±1 vs. saline+saline=103±6). Values obtained from the group infused only with the selective antagonist (D-Lys^3-GHRP-6+saline) were similar to those from the saline+saline group (D-Lys3-GHRP-6+saline=95±13 vs. saline+saline=103±6), indicating that the antagonist by itself did not modify the threshold to generate LTP. When Ghr was infused in animals pretreated with the antagonist, the Ghr effect upon the threshold was not evident, indicating that this effect was mediated by GHS-R1a activation (D-Lys3-GHRP-6+Ghr=85±5 vs. saline+saline=103±6) ($F_{(3, 17)}$=34.9, $p<0.05$) (Fig. 2d, e).

In another set of experiments, it was also studied the response of the slices of different groups to the classical stimulation paradigm (100 Hz). In all groups, LTP was generated and no differences were observed in the magnitude of the generated LTP (data not shown).

Effect of Ghr on 4-AP-evoked release of endogenous glutamate from hippocampal synaptosomes

In order to explore if Ghr effects were related to presynaptic Glu release, we used 4-AP-evoked-glutamate release from hippocampal synaptosomes. It is well known that synaptosomes retain the morphological features and chemical composition of the presynapses. Figure 3a is a representative recording (temporal pattern) of evoked-glutamate release from hippocampal synaptosomes in absence (control) or presence of Ghr (3 nM). As it can be seen, Ghr administration increased the evoked-glutamate release when compared with the control group along the whole period recorded. The effect of different doses of the peptide upon Glu release was also tested. Results were expressed as percent of the evoked release compared with the control, considering the control as 100%. At concentrations of Ghr of 0.03, 3, and 300 nM, the percent of Glu release reached to 114.3±5.5, 128.0±4.1, and 130.8±4.7%, respectively ($F_{(5, 15)}$=20.5, $p<0.05$) (Fig. 3b). Ghr significanlty increased evoked-glutamate release in a dose-dependent manner, reaching a maximum at Ghr concentrations of 3 and 300 nM (approximately +30% respect to the control values) and showing a significant decrease in the percent of Glu release at the highest dose tested (30 µM), suggesting that Ghr may have a role in regulating excitatory synaptic transmission; it also supports the hypothesis that Ghr could act at presynaptic level.

Furthermore, when the area under the curve (AUC) was measured, representing the total Glu released during the experiment, a significant increase was observed in Ghr at 0.03, 3, and 300 nM (118±2.9, 119±3.9, and 121±2.3%, respectively) compared with the control group (100%) ($F_{(5, 15)}$= 19.2, $p<0.05$).

Impact of intra-hippocampal Ghr administration on the number of NR2B-immunoreactive cells

Considering the critical requirement of NR2B-containing NMDAR for memory facilitation and LTP induction, we

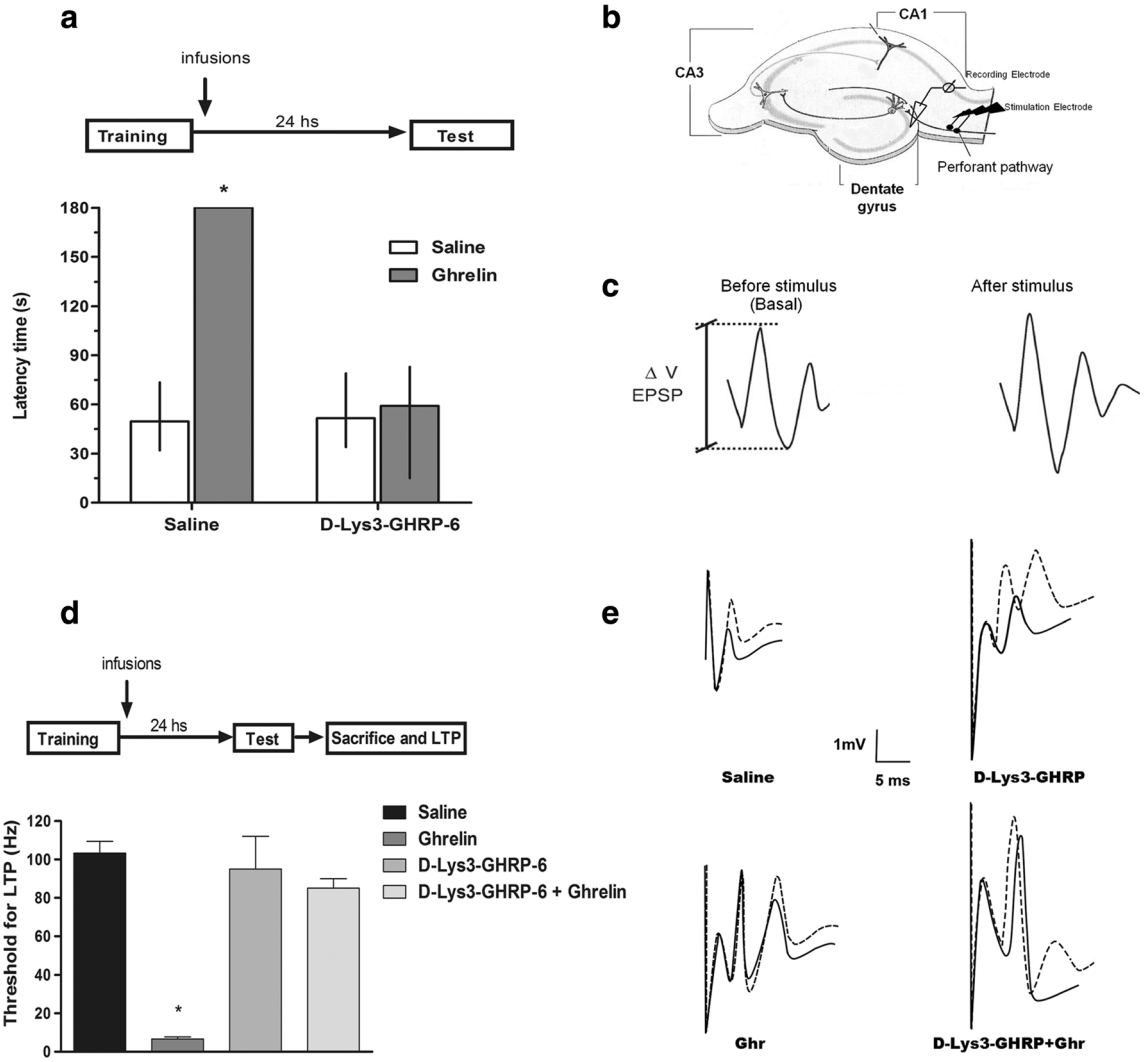


Fig. 2 Intra-hippocampal Ghr administration in presence of the antagonist of Ghr receptor, D-Lys3-GHRP-6. (**I**) Effect on memory retention in SDT (**a**). The treatments were performed immediately posttraining, and the latency time to step down was quantified 24 h later for long-term memory. The animals were injected with saline+saline, saline+Ghr, D-Lys3-GHRP-6+saline, or with D-Lys3-GHRP-6+Ghr. The results are expressed as medians of latency time with the respective inter-quartile range. N=8–10 animals/group. *$p<0.01$, significant differences related to the control animals (saline). (**II**) Effects upon electrophysiological parameters. **b** Hippocampal slice picture indicating the position of stimulation and recording electrodes. **c** Picture shown how measurements are taken in a typical example of field excitatory postsynaptic potential (*EPSP*) obtained in the hippocampal dentate gyrus before and after tetanus showing. **d** Bar graphs showing threshold to generate LTP in slices from rats exposed to different treatments. *Asterisk*, significantly different from the control animals. *Bars* indicate threshold means (Hz) and *vertical lines* ±SE. **e** EPSP sample traces for different groups before (*full line*) and after (*dotted line*) effective tetanus. N=5–6 animals/group. *$p<0.05$ compared with saline group. *Bars* represent means±SE

explored if NR2B-subunit expression in the hippocampus was altered after in vivo Ghr administration.

Immunohistochemical experiments Figure 4a, b indicates the cannula localization for Ghr administration and position at the CA1 area of hp, respectively. Figure 4c shows photomicrographs of NR2B-IR cells in hippocampal slices obtained 24 h after each treatment. Our results revealed that hippocampal slices from animals trained and infused with Ghr (Ghr group) show significant increase in the number of positive cells in CA1 and DG when compared with the control (animals trained and infused with saline solution) and naïve (animals without surgery, infusion, or training) groups (Ghr_{CA1}=171±2 vs. $control_{CA1}$=139±6 vs. $naïve_{CA1}$=149±7; Ghr_{DG}=52±3 vs. $control_{DG}$=37±3 vs. $naïve_{DG}$=38±5; $F_{CA1\ (2,\ 9)}=11.6$; $F_{DG\ (2,\ 9)}=7.4$; $p<0.05$) (Fig. 4d). The naïve group was included in order to discard the fact that changes in the number of NR2B-IR cells were consequence of the experimental procedure (surgery and/or training). No differences were observed between the naïve and control groups, indicating that basal

NR2B-subunit expression was not affected by the experimental procedure (Fig. 4d). Then, the increased number of NR2B-IR cells induced by Ghr may account, at least in part, for the increased hippocampal excitability and in consequence, contribute to the improved performance in the behavioral memory paradigm.

Effects of intra-hippocampal Ghr administration in animals pretreated with Ro 25-6981

In order to add functional information about participation of the NR2B-containing NMDAR in Ghr effects, in this set of experiments we studied the behavioral and electrophysiological effects of the peptide (3 nmol/μl) in animals previously infused with the NR2B-specific antagonist, Ro 25-6981, 5 μg/μl (this dose of the antagonist was chosen because it was able to inhibit the expression of memory retention in SDT as well as the LTP induction).

Behavioral experiments Memory retention was analyzed by measuring the latency time to step down in animals treated with saline+saline, saline+Ghr, Ro 25-6981+saline, and Ro 25-6981+Ghr (Fig. 5a). Previous infusion of the antagonist (Ro 25-6981+saline group), significantly decreased the latency time in SDT in relation to the control group (saline+saline), indicating an impairment in memory retention induced by the antagonist. Administration of Ghr 3 nmol/μl (saline+Ghr

Fig. 4 Hippocampal expression of NR2B subunits after Ghr treatment. **a** ▶ Schematic drawings of coronal sections of the rat brain (according to the atlas of Paxinos and Watson) indicating the cannula localization at the CA1 area of the hippocampus. The cannulae were implanted bilaterally into the hippocampus. Seven days after surgery, the behavioral training was made, Ghr or saline was infused, the animals were killed, the hippocampal slices were obtained, and the immunoreactivity in the CA1 and DG areas were determined. The millimeters anterior to the bregma are indicated in each drawing. **b** Photomicrograph showing the cannula's position; only the injection side in one hemisphere is represented. **c** *Photomicrograph with scale bar*=100 μm. **d** *Photomicroghraps* and *bars graph* showing immunoreactivity of NR2B-positive cells in slices from the control and Ghr-treated animals in two different hippocampal areas (CA1 and DG). The experimental groups were: naïve (without infusion, without training), control (infused with saline solution), Ghr (infused with Ghr), and negative control (hp slices without the primay antibody). N=4 animals/group. *$p<0.05$, significant differences related to the control group

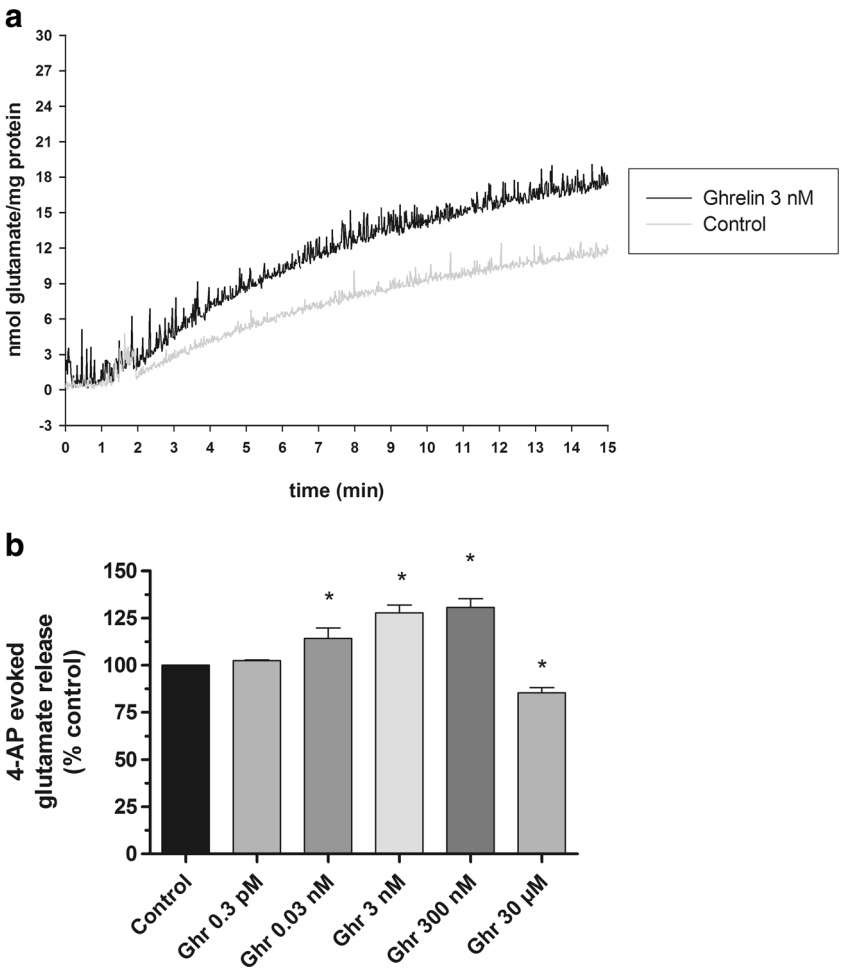
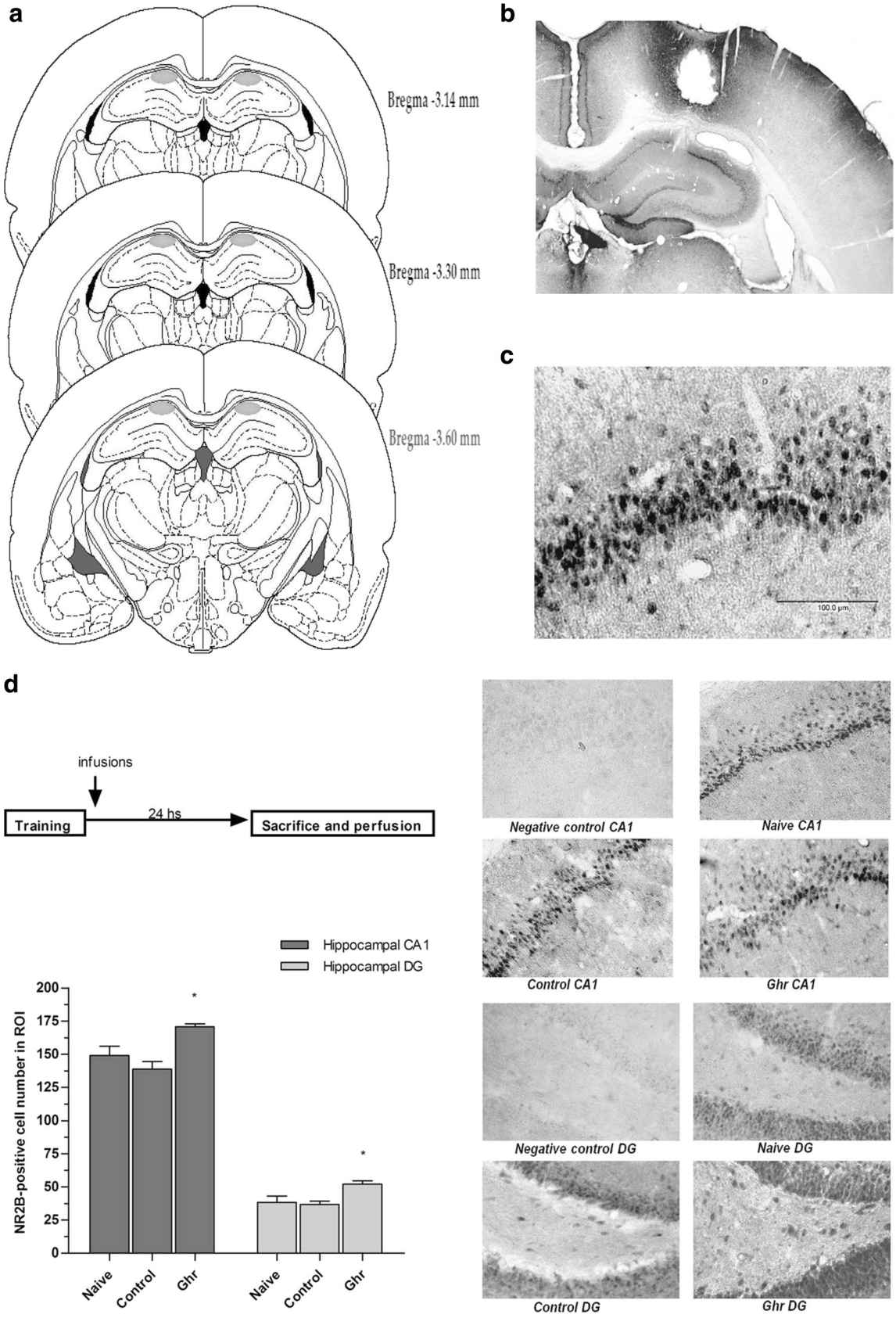


Fig. 3 Effect of Ghr on 4-AP-evoked release of endogenous glutamate from the hippocampal synaptosomes. **a** Representative recording of Ghr (3 nM) effect on the release of glutamate from hippocampal synaptosomes during 15 min (*black* and *gray* traces, with or without Ghr, respectively). **b** The *bars* represent the Ghr-induced glutamate release with different doses of the peptide. Results were expressed as percent of the evoked release compared with the control, considering the control as 100 %. The mean±SEM was obtained from of the four independent experiments performed in triplicate. *$p<0.05$, a value that is significantly increased over control condition (without ghrelin) as determined by one-way ANOVA followed by Newman–Keuls post hoc test


a
Bregma -3.14 mm
Bregma -3.30 mm
Bregma -3.60 mm
b
c
100.0 µm
d
infusions
Training
24 hs
Sacrifice and perfusion
Hippocampal CA1
Hippocampal DG
NR2B-positive cell number in ROI
200
175
150
125
100
75
50
25
0
Naive
Control
Ghr
Naive
Control
Ghr
Negative control CA1
Naive CA1
Control CA1
Ghr CA1
Negative control DG
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Ghr DG

group) increased the latency time in the SDT, as it was previously described in Fig. 1. Nevertheless, when Ghr was infused in animals previously treated with Ro 25-6981 (Ro 25-6981+Ghr group), values obtained for latency time were similar to the control group, but they were significantly lower than saline+Ghr group ($H_{(1)}$=19.73; p<0.01), suggesting that Ghr was able to revert the deleterious effect of the antagonist.

Electrophysiological experiments In this set of experiments, we analyzed possible differences in LTP generation by using the classical tetanization paradigm (second protocol) by comparing the amplitude of EPSP related to basal EPSP (% EPSP) in the different experimental groups after HFS (Fig. 5b). A significant difference was observed when saline+saline, saline+Ghr, and Ro 25-6981+Ghr groups were compared with Ro 25-6981+saline group ($F_{(3, 22)}$=4.98; p<0.05), indicating that LTP generation was impaired in slices from rats pretreated with Ro 25-6981+Saline. Post hoc Newman–Keuls test revealed no differences in the percent of EPSP between saline+saline, saline+Ghr, and Ro 25-6981+Ghr groups.

Considering that LTP generation was impaired in Ro 25-6981+saline group and no differences in the degree of EPSP potentiation were observed between other groups, we tested if there were differences in the frequency values necessary to

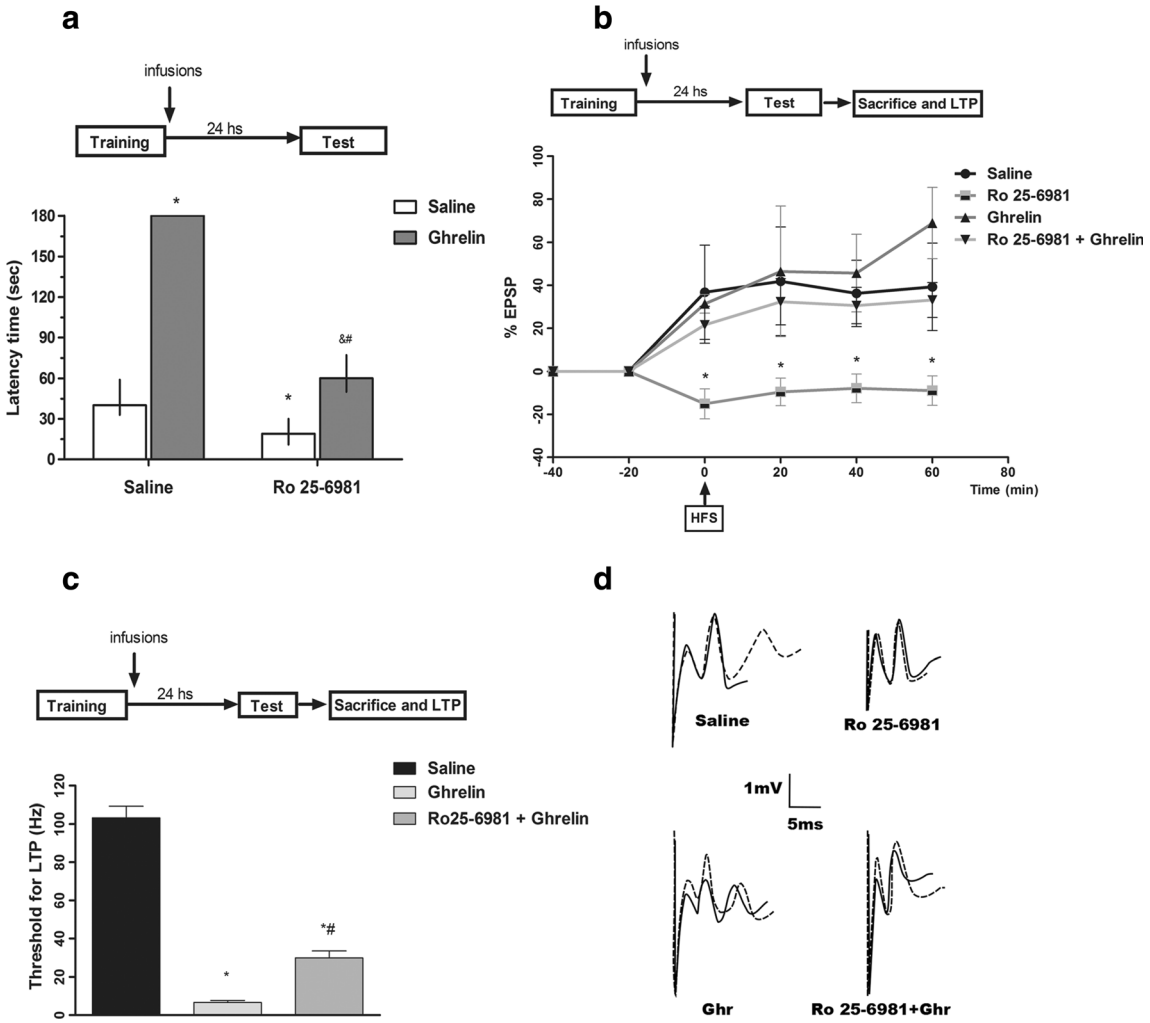

Fig. 5 Effects of intra-hippocampal Ghr administration in animals pretreated with Ro 25-6981. (I) Effect on memory retention in SDT (**a**). The rats were trained in the SDT 7 days after surgery. Ghr (3 nmol/μl) was injected immediately after training; Ro 25-6981 (5 μg/μl) was infused 5 min before Ghr. Twenty-four hours after, animals were tested in the SDT and memory retention was evaluated. Data are expressed as the median and inter-quartile range. N=10–12/group. *p<0.01, significantly different from vehicle alone (saline); $^{\#}p$<0.01, significantly different from Ghr-treated rats; &p<0.01, significantly different from Ro 25-6981-treated rats. (II) Effects upon electrophysiological parameters. **b** Percentage of increase in the EPSP in the different treatments. *Asterisk*, significantly different from basal EPSP and saline and Ghr and Ghr+Ro 25-6981 groups. **c** *Bar graphs* showing threshold to generate LTP in slices from rats exposed to different treatments. *Bars* represent means± SE. *Asterisk*, significantly different from saline animals. *Bars* indicate threshold means (Hz) and *vertical lines* ±SE. **d** EPSP sample traces for different groups before (*full line*) and after (*dotted line*) effective tetanus. N=6/group. *p<0.05, significantly different compared with the saline group

induce LTP ("threshold") in DG of hippocampal slices from groups in which LTP was generated by the second protocol (saline+saline, saline+Ghr, Ro 25-6981+saline, and Ro 25-6981+Ghr) (Fig. 5c). As it can be seen, a significant reduction in the stimulating frequency values to generate LTP was observed in slices from saline+Ghr-infused animals compared with saline+saline, consistent with our previous reports. When Ghr was infused in animals pretreated with the antagonist Ro 25-6981 (Ro 25-6981+Ghr group), the frequency values to induce LTP were significantly increased compared with saline+Ghr group but significantly lower than saline+saline group (Ro 25-6981+Ghr=30±3.6 vs. saline+saline=103.3±6.1 vs. saline+Ghr=6.7±1.0 Hz; $F_{(2,\ 15)}=146.2$; post hoc Newman–Keuls test, $p<0.05$).

Discussion

These results provide the first evidence that Ghr increased Glu release from hippocampal synaptosomes, indicating a presynaptic effect mediated by Ghr. In addition, we demonstrated that the intra-hippocampal Ghr administration increased NR2B-subunit expression in hippocampal CA1 and DG and also reversed the deleterious effects of the NR2B-specific antagonist, Ro-25-6981, upon memory consolidation and LTP generation in DG. The behavioral and electrophysiological results are likely to be the consequence of the specific stimulation of GHS-R1a, since administration of the selective antagonist, D-Lys3-GHRP-6, prior to the peptide, prevented the Ghr-induced behavioral effects and the enhancement in hippocampal synaptic plasticity, previously observed.

Previous results from our laboratory demonstrated that Ghr facilitates memory when it was administered systemically or intra-ventricularly (Carlini et al. 2002, 2004, 2008). Furthermore, the intra-hippocampal injection improved memory retention in a dose-dependent manner and it was correlated to a decrease in the threshold to LTP induction in hp (Carlini et al. 2010). These finding suggested that Ghr improved memory acquisition and/or consolidation by modulating hippocampal molecular and/or cellular signaling leading to an increased excitability in this brain area.

It is well known that drug administration after training is a frequently used method to influence memory (to impair or to enhance) consolidation without affecting either acquisition or memory retrieval (Medina et al. 2008). The results of the present research showed that the facilitation in memory consolidation induced by Ghr was observed only when Ghr was administered immediately after training session but not 15 or 60 min after training (see Fig. 1). These findings indicate that Ghr exert its effects in a particular temporal window when it is administered posttraining, suggesting that the peptide probably modulates the first biochemical events of the memory cascade. These early events may also trigger late events necessary to maintain LTP and long-term memory effects, since Ghr effects in long-term memory were observed 24 h after training.

The fact that GHS-R1a are highly expressed in the hp reinforces the idea that Ghr could be a prominent neuromodulator of hippocampal neurons activity (Cuellar and Isokawa 2011; Zigman et al. 2006). Then, in another set of experiments, we studied if the Ghr-induced behavioral and electrophysiological changes were mediated by direct activation of hippocampal GHS-R1a. Our results showed that both electrophysiological and behavioral peptide- induced effects where prevented when the Ghr receptor is blocked, evidencing a clear participation of GHS-R1a in the mentioned effects.

Synaptosomes are subcellular membranous structures that are formed during the mild disruption of the brain tissue and retain the morphological features and chemical composition of the presynapses (Nicholls 2003). It has been demonstrated that crude synaptosomes (P2 fraction) purified from adult rat hp are enriched in GSH-R1a; these receptors are located in the vicinity of glutamatergic synapses, suggesting that Ghr may have a role in regulating excitatory synaptic transmission (Ribeiro et al. 2014). Taking into account our previous results and the above mentioned findings, it could be hypothesized that Ghr modulates Glu release at the presynaptic level. Then, we measured possible changes induced by Ghr in evoked-glutamate release from hippocampal synaptosomes. The above mentioned hypothesis was supported by the fact that Ghr increased the evoked-glutamate release in a dose-related manner. Considering the above mentioned findings, it is reasonable to believe that the mechanisms underlying Ghr effects could be similar in different brain areas. The described mechanisms by which Ghr mediates the effects upon feeding at the arcuate nucleus (Arc) of the hypothalamus is in accordance with this hypothesis. It implicates GHS-R1a activation, modulation of electrical activity in cells related to feeding regulation, such as the agouti-related peptide neurons (AGRP), and increase in Glu release at the presynaptic level (Yang et al. 2011). In accordance to the abovementioned results in the Arc, our results seem to indicate that Ghr exerts some effects in hp acting at the presynaptic level.

Glu is an important neurotransmitter related to memory processes, since the biochemical memory cascade and LTP induction are initiated by Glu release from the presynaptic membrane followed by the activation of NMDA and AMPA receptors at postsynaptic level. It has been demonstrated that differential assembly of the subunits of the NMDAR is thought to result in channels with different functional properties. Furthermore, genetic deletion of NR2B-subunits of the NMDAR in the hp or forebrain (specific knockout of NR2B animals) result in profound memory deficits and impaired LTP (Sprengel et al. 1998; von Engelhardt et al. 2008). Oppositely, numerous animal models that feature elevated NR2B levels

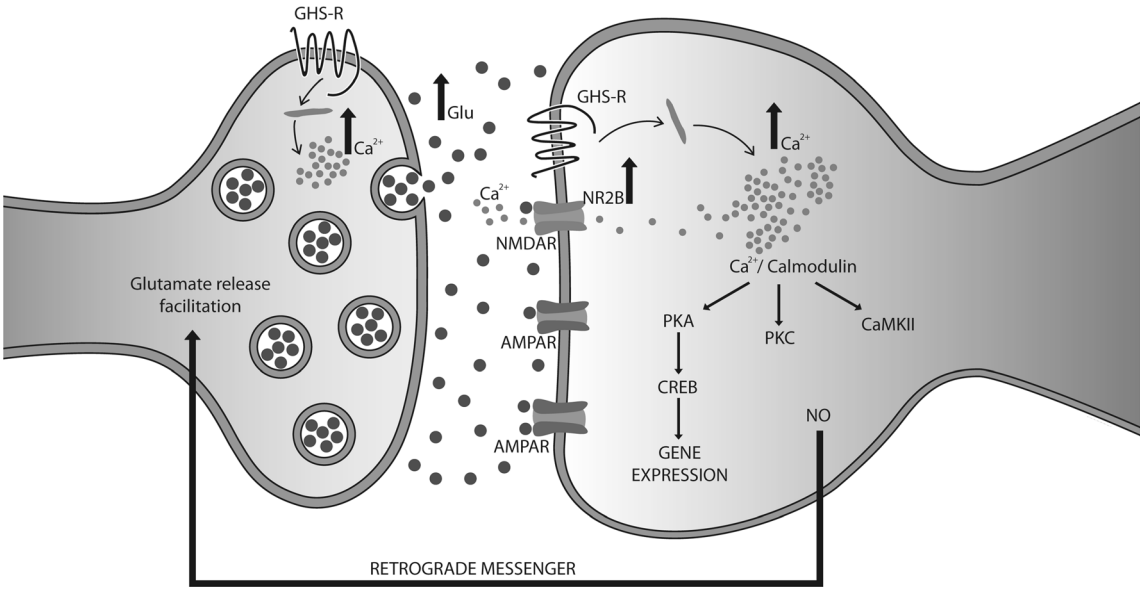

Fig. 6 Hypothesis about the Ghr effects upon early events of the memory consolidation: Ghr binds its receptors at pre- and postsynaptic levels. At presynaptic level, Ghr stimulates Glu release, probably by increasing $[Ca^{+2}]_i$ release. At postsynaptic level, the Glu released activates NMDAR while Ghr also mediates direct effects by activating its receptors. Thus, Ghr signaling probably cross-talks with the components of the early biochemical cascade of memory and LTP synergizing the $[Ca^{2+}]_i$ increase and/or the activation of CaMKII which binds to NR2B subunits and activates NOS/NO pathway (Carlini et al. 2010), contributing to NR2B-subunit up-regulation that may improve LTP and memory retention. PKA phosphorylation of NR2B subunits could also be related to the potentiation of NMDAR-Ca^{2+} signaling at synapses (Murphy et al. 2014; Flores-Barrera et al. 2014)

via altered synthesis, transport, or degradation exhibit improved synaptic plasticity and memory (Lee and Silva 2009) (Crair and Malenka 1995; Tang et al. 1999). Hence, targeting NR2B and its regulatory machinery has been singled out as an attractive approach for cognitive enhancement (Bibb et al. 2010; Collingridge et al. 2013). Genetic over-expression of NR2B-subunits in the mouse forebrain can lead to larger hippocampal LTP and enhanced learning and memory function as tested in many different memory tasks (Tang et al. 1999, 2001; Cao et al. 2007; Jacobs and Tsien 2012; Cui et al. 2011). Similar results in memory and LTP enhancement were observed in NR2B-subunit over-expression in rats, pointing out that the beneficial effects of NR2B are conserved in multiple animal species (Wang et al. 2009). In addition, the levels of NR2A or NR2B expression in the cortex and hp can also be dynamically modulated by individual experiences (i.e., enriched environment or social interactions) (Rauner and Kohr 2011). In this work, we also examined if changes in functional properties in hp induced by Ghr could be a consequence of increased NR2B-subunit expression. In accordance to the above mentioned findings, an increase in NR2B-subunit expression induced by the peptide could explain the enhancement of long-term memory and the persistence of the LTP. Our results showed that acute Ghr administration increases NR2B-subunit expression in hippocampal CA1 and DG 24 h after Ghr administration, probably contributing to both the increased LTP generation and long-term memory in the SDT.

In another set of experiments, Ro 25-6981, a NR2B-containing NMDAR-specific antagonist, was used in a dose able to inhibit the memory retention in SDT and LTP generation, in order to add functional information related to NR2B-containing NMDAR participation in Ghr effects. When Ghr was administered in animals pretreated with Ro 25-6981, the deterioration induced by the antagonist was reverted, and hippocampal excitability restored to values similar to the saline group in both behavioral paradigm and LTP generation. The fact that Ghr administration in animals pretreated with the NR2B antagonist restored the behavioral and electrophysiological parameters at levels similar to the control group (saline group) lead us to hypothesize that the effects observed may be a consequence of increased expression of NR2B-containing NMDAR.

It has also been demonstrated that calcium influx through the NMDAR induced an initial Ca^{2+}/CaM (calmodulin) activation that activates CaMKII (Ca^{2+}/calmodulin (CaM)-dependent protein kinase II) which, in turn, is responsible for the amplification of the signals initiated by activation of the NMDAR during the onset of plastic events. Therefore, it is noteworthy that CaMKII binds to the NR2B-subunit of the NMDAR and induces LTP in excitatory synapses (Bayer et al. 2006; Isokawa 2012). Also, it has been demonstrated that PKA phosphorylation of NR2B-subunits of the NMDAR is critical to the potentiation of NMDAR-Ca^{2+} signaling at synapses (Murphy et al. 2014; Flores-Barrera et al. 2014).

In addition, recent reports suggested that Ghr phosphorylates NR2B-subunits and indirectly enhances the NMDAR function (Isokawa 2012). The molecular mechanism that mediates the Ghr-induced-NR2B up-regulation could not be elucidated from the experiments presented in this work. Thus, we believe that changes in the NR2B-subunits binding to CaMKII could be the most probable scenario for the mechanisms responsible for the increase in NR2B-subunits. However, we cannot rule out other mechanisms such as the induction of de novo synthesis, the decreased degradation of NR2B via distinct intracellular signaling pathways (Hawasli et al. 2007) or changes in the trafficking of Glu receptors from the cytoplasm to synaptic sites.

It is well known that increased $[Ca^{+2}]_i$ is a common requirement to initiate most forms of synaptic plasticity including LTP (Isokawa 2012). The NMDAR activation induces Ca^{+2} influx through its associated ion channel, activating different enzymes and protein kinases (Izquierdo and Medina 1997). GHS-Rs transduction mechanism in several tissues involves Ca^{+2} influx in response to their activation. Thus, for example, Ghr increased $[Ca^{+2}]_i$ levels in cells from the Arc (Kohno et al. 2003, 2008; Yang et al. 2011).

In conclusion, the results presented here reinforce the idea that Ghr could modulate early events of memory consolidation in hp probably by increasing the $[Ca^{+2}]_i$ levels, enhancing Glu release which, in turn, activates the NMDAR and stimulate different molecular pathways that contribute to the maintenance of LTP and long-term memory retention. CaMKII is a target in hippocampal synaptic plasticity mediated by the NMDAR. The up-regulation of NR2B-subunits probably could be a consequence of an increased $[Ca^{+2}]_i$ level, and increased activation of CaMKII. Our hypothesis about the Ghr effects upon events of the memory consolidation is represented in Fig. 6: Ghr binds its receptors at pre- and postsynaptic levels. At presynaptic level, Ghr stimulates Glu release, probably by increasing $[Ca^{+2}]_i$ release. At postsynaptic level, the Glu released activates NMDAR while Ghr also mediates direct effects by activating its receptors. Thus, Ghr signaling probably cross-talks with the components of the early biochemical cascade of memory and LTP synergizing the $[Ca^{2+}]_i$ increase and/or the activation of CaMKII which binds to NR2B-subunits and activates NOS/NO pathway (Carlini et al. 2010), contributing to NR2B-subunit up-regulation that may improve LTP and memory retention. PKA phosphorylation of NR2B- subunits could also be related to the potentiation of NMDAR-Ca^{2+} signaling at synapses (Murphy et al. 2014; Flores-Barrera et al. 2014).

The findings presented in this work give place to new perspectives for the understanding of the potential therapeutic role of Ghr in pathologies induced by dysfunction of NR2B-subunits containing NMDAR, such as Alzheimer, Parkinson, Schizophrenia, and other cognitive disorders.

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