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Ghrelin induced memory facilitation implicates nitric oxide synthase activation and decrease in the threshold to promote LTP in hippocampal dentate gyrus

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ABSTRACT

Although the hypothalamus has been long considered the main ghrelin (Ghr) target organ mediating orexigenic effects, recently it has been shown that in-vivo Ghr hippocampus administration improves learning and memory in the inhibitory avoidance paradigm. However, the possible mechanisms underlying this memory facilitation effect have not been clarified. Given that the biochemical memory cascade into the hippocampus involves nitric oxide (NO) synthesis via NO synthase (NOS) activation, we investigated 1) if Ghr administration modulated NOS activity in the hippocampus; and 2) if hippocampal NOS inhibition influenced Ghr-induced memory facilitation, using a behavioral paradigm, biochemical determinations and an electrophysiological model. Our results showed that intra-hippocampal Ghr administration increased the NOS activity in a dose dependent manner, and reduced the threshold for LTP generation in dentate gyrus of rat hippocampus. Moreover, pre-administration of NG-nitro-L-arginine (L-NOArg) in the hippocampus partially prevented the Ghr-induced memory improvement, abolished the increase in NOS activity, and prevented the decreased threshold to generate LTP induced by Ghr. These findings suggest that activation of the NOS/NO pathway in hippocampus participates in the effects of Ghr on memory consolidation and is related with plastic properties of the hippocampal three-synaptic loop.

1. Introduction

Ghrelin (Ghr) is a 28 amino acid acylated peptide, which is synthesized both peripherally and centrally. It is known that Ghr participates in the modulation of several processes related to energy homeostasis, gastrointestinal functions, anxiety-like behavior and growth hormone releasing activity [1]. This peptide is an endogenous ligand for the growth hormone secretagogue receptor (GHS-R), with seven transmembrane domains, and coupled to G protein [2,3].

Signal transduction involves activation of phospholipase C (via G protein), generation of inositol three-phosphate and diacyl glycerol, and an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) [3]. In the central nervous system, GHS-Rs are mainly expressed in the hypothalamus, and mediate the orexigenic effects [4,5]. However, they are also, expressed in extrahypothalamic structures such as the hippocampus [6,7], a structure related to learning and memory [8].

It has been demonstrated the hippocampus involvement in learning and memory processes [9]. The one-trial step-down inhibitory avoidance in rodents has long been used as a model for biochemical and pharmacological studies of memory [10]. It has been previously reported that the main events during consolidation in a one-trial avoidance coincide with the signaling pathway activated during the LTP induction in hippocampal CA1 [11,12], and are initiated by activation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and N-methyl D-aspartate (NMDA) glutamate receptors [8,13,14]. This activation induces increases of $[Ca^{2+}]_i$, followed by activation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII), thus increasing nitric oxide synthase (NOS) activity and cyclic guanosine monophosphate (cGMP) levels, with among activation of other intracellular signaling pathways [13,15].

Nitric oxide (NO) is a gas membrane-permeable molecule, which is currently recognized as a neuronal messenger synthesized by the enzyme NOS. The latter, converts arginine into equimolar quantities of citrulline and NO [16,17]. NO acts as a retrograde messenger [18], and promotes pre- and post-synaptic effects via mainly cGMP production, but, in less extent, it can promote intracellular signaling through cAMP production or s-Nitrosilation of proteins [18–20]. Some forms of induced LTP can be eliminated or significantly reduced by application of NOS inhibitors, indicating a possible role of the NOS/NO pathway in LTP induction and maintenance [21–23]. Moreover, NOS stimulators or inhibitors can affect some forms of learning and memory [24].

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We have recently shown that Ghr administration, either intracerebroventricularly [25] or directly into brain areas such as the hippocampus, amygdala or dorsal raphe nucleus, enhanced memory consolidation in a one-trial step-down inhibitory avoidance in rats in a dose-dependent manner [26]. Furthermore, it has been demonstrated that if the peptide is peripherally administered, it can reach the hippocampus and promotes LTP in CA1 [27]. However, the precise molecular mechanisms underlying the Ghr effects on memory in the hippocampus are still unclear.

Thus, in the present study, we investigated if Ghr administration modulates the step of the early biochemical memory cascade that involves changes the hippocampal NOS activity, and whether the inhibition of NOS in the hippocampus affects the Ghr induced memory facilitation, using a behavioral paradigm, biochemical determinations and an electrophysiological model.

2. Results

2.1. Hippocampal NOS activity in Ghr administered animals

Fig. 1 shows the hippocampal NOS activity measured after Ghr administration in rats. For one set of experiments (Panel A), animals were administered with artificial cerebrospinal fluid (ACSF) or different doses of Ghr (0.03, 0.3 and 3.0 nmol/μl). Our results showed that only animals administered with Ghr 3.0 nmol/μl (Ghr 3.0 nmol/μl = 1.40 ± 0.25 pmol/mg protein) induced a significant increase in NOS activity when compared to ACSF treated (control) animals (0.27 ± 0.03 pmol/mg protein) (F (3, 22) = 12.65, $p \leq 0.05$). On the other hand (Panel B), when the animals received the step-down test training (trained animals), Ghr induced significant increases in NOS activity at doses of 0.3 and 3.0 nmol/μl (Ghr-trained 0.3 nmol/μl = 2.56 ± 0.19; Ghr-trained 3.0 nmol/μl = 3.39 ± 0.31 pmol/mg protein) in comparison to control animals (ACSF-trained = 1.82 ± 0.17 pmol/mg protein) (F (3, 29) = 10.32, $p \leq 0.05$).

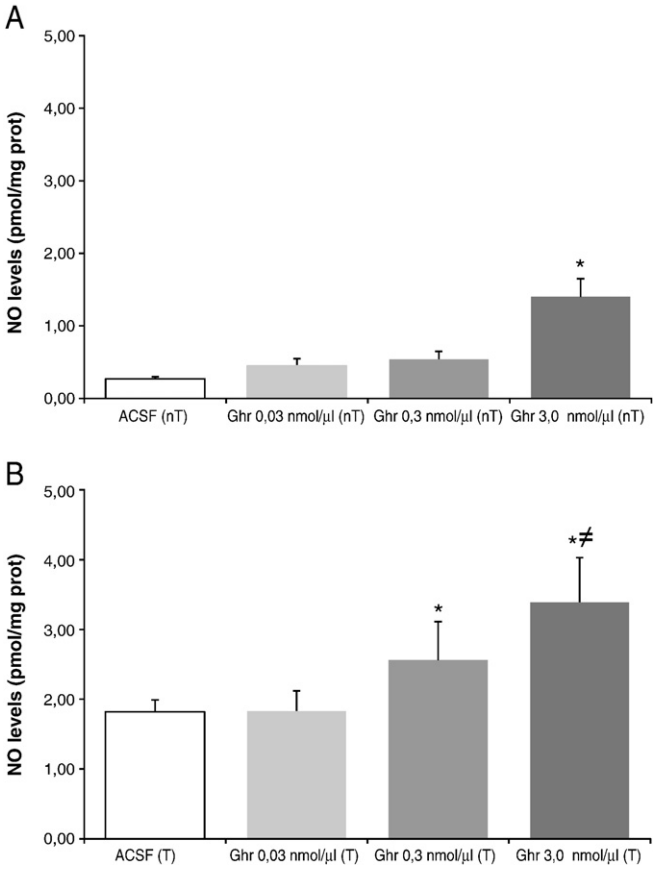

Fig. 1. Ghr effect (0.03, 0.3 and 3.0 nmol/μl) on NOS activity. Panel A shows the effect of Ghr administration on NOS activity in the untrained animal group (nT), with Panel B showing the effect of Ghr administration on NOS activity in the trained animal (T) group in the step-down paradigm. The results are expressed as means in NO pmol/mg protein ± SEM. N = 5–8 animals/group. *: significant differences related to ACSF animals, $p \leq 0.05$. #: significant differences related to Ghr 0.3 nmol/μl, $p \leq 0.05$.

In order to explore the possibility that the increase in NOS activity observed could be related to the activation of constitutive NOS (which is Ca^{2+} dependent [28], or the inducible NOS, the NOS activity was also measured in a free Ca^{2+} buffer in the trained animal groups (Ghr 3.0 nmol/μl-trained and ACSF-trained). The results showed that NOS activity in a free Ca^{2+} buffer did not differ between groups (Ghr 3.0 nmol/μl-trained = 0.60 ± 0.14 pmol/mg protein vs. ACSF = 0.62 ± 0.09 pmol/mg protein) (F (1, 10) = 0.00, $p > 0.05$).

2.2. Effect of NOS inhibition on memory retention and NOS activity in hippocampus of Ghr treated animals

Fig. 2 shows the Ghr effect upon behavioral performance in a step-down test 1 h after training for different animal groups pretreated with L-NOArg. Administration of L-NOArg 2 μg/μl decreased the latency time when compared to control animals treated with ACSF. However, when Ghr (0.3 and 3.0 nmol/μl) was administered in animals previously treated with the inhibitor, the latency was similar to those obtained in control animals (H (5, 52) = 45.06, $p \leq 0.05$).

Fig. 3 shows the Ghr effect on long-term memory 24 h after training in animals pretreated with L-NOArg. Animals injected with L-NOArg and ACSF showed a significant decrease in the latency in comparison to control animals (H (5, 52) = 41.86, $p \leq 0.05$). Moreover, the latency time in animals treated with L-NOArg and Ghr 0.3 nmol/μl was similar to the control animals ($p > 0.05$).

Fig. 4 shows the effect of Ghr administration on NOS activity in trained animals pretreated with the NOS inhibitor. The two way ANOVA test revealed a significant interaction between treatment with Ghr and L-NOArg (F (2, 44) = 3.63, $p \leq 0.05$), and a significant effect of L-NOArg treatment (F (1, 44) = 110.56, $p \leq 0.05$) and Ghr treatment (F (2, 44) = 46.00, $p \leq 0.05$). The administration of L-NOArg and ACSF decreased the NOS activity in comparison to control animals (ACSF and ACSF) ($p \leq 0.05$). The trained animals treated whit ACSF prior to the Ghr administration (0.3 and 3.0 nmol/μl) exhibited a significant increased the NOS activity in comparison to control animals ($p \leq 0.05$). However, Ghr administration in trained animals pretreated

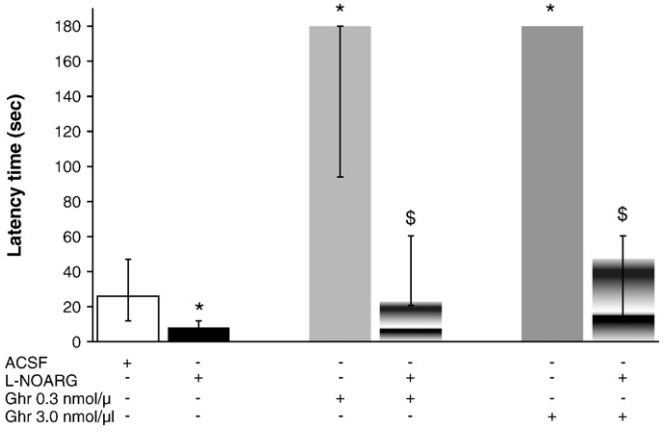

Fig. 2. Ghr effect on short-term memory retention in animals pretreated with NOS inhibitor. Animals were administrated with L-NOArg (2 μg/μl) and Ghr (0.3 and 3.0 nmol/μl) immediately after training and evaluated 1 h later for short-term memory. The results on memory retention are expressed as medians with the respective inter-quartile range. N = 8–10 animals/group. *: significant differences related to control animals (ACSF), $p \leq 0.05$. $: significant differences related to animals treated with L-NOArg alone, $p \leq 0.05$.

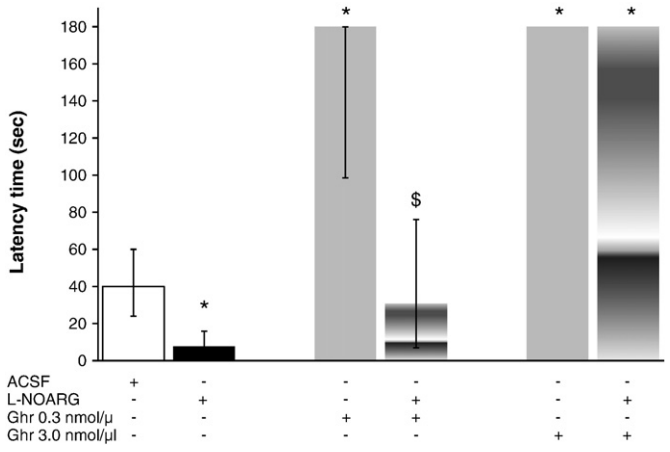

Fig. 3. Ghr effect on long-term memory retention in animals pretreated with NOS inhibitor. Animals were administrated with L-NOArg (2 µg/µl) and Ghr (0.3 and 3.0 nmol/µl) immediately after training and evaluated 24 h later for long-term memory. The results on memory retention are expressed as medians with the respective inter-quartile range. $N = 8–10$ animals/group. *: significant differences related to control animals (ACSF), $p \leq 0.05$. $: significant differences related to animals treated with L-NOArg alone, $p \leq 0.05$.

with L-NOArg did not induce significant changes in NOS activity when compared to control animals, $p > 0.05$ (L-NOArg prior to Ghr administration vs. ACSF).

2.3. Effect of NOS inhibition in Ghr treated animals on the threshold to generate LTP in hippocampal dentate gyrus

As can be seen in Fig. 5B the *extra-cellular recordings obtained in* the hippocampal dentate gyrus showed a significant reduction in the threshold to generate LTP: rats treated with Ghr 0.3 and 3 nmol/µl compared to slices from ACSF pretreated rats (ACSF vs. Ghr 0.3 nmol/µl = 87 ± 9.6 vs. 6 ± 1.1 Hz and ACSF vs. Ghr 3 nmol/µl = 87 ± 9.6 vs. 9 ± 3.3 Hz; unpaired *t*-tests, both $p < 0.05$).

When Ghr (0.3 nmol/µl) was administered in animals previously treated with the NOS inhibitor, similar threshold values to generate LTP were observed in this group when compared to control group (L-NOArg + Ghr 0.3 nmol/µl vs. ACSF + ACSF = 80 ± 14.1 vs 87 ± 9.6 Hz, unpaired *t*-test, $p > 0.05$). Significant differences were observed between animals that received ACSF + Ghr when compared to animals pretreated with the inhibitor and Ghr (ACSF + Ghr 0.3 nmol/µl vs.

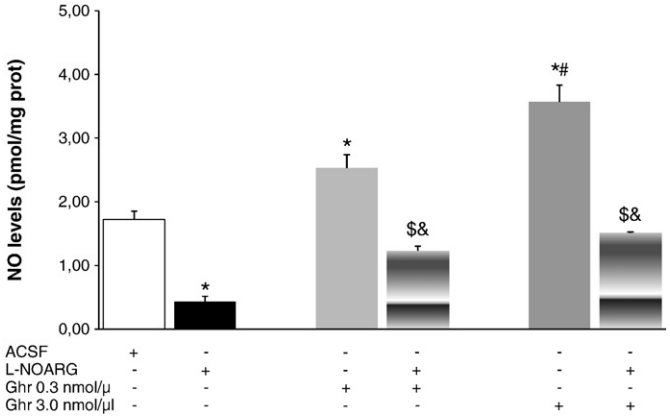

Fig. 4. Ghr effect on NOS activity in trained animals pretreated with NOS inhibitor in a step-down paradigm. The results are expressed as means in NO pmol/mg protein ± SEM. $N = 6–10$ animals/group. *: significant differences related to control animals (ACSF), $p \leq 0.05$. #: significant differences related to Ghr 0.3 nmol/µl, $p \leq 0.05$. $: significant differences related to animals treated with L-NOArg alone, $p \leq 0.05$. &: significant differences related to animals treated with Ghr alone, $p \leq 0.05$.

L-NOArg + Ghr 0.3 nmol/µl = 6 ± 1.1 vs. 80 ± 14.1 Hz, unpaired *t*-tests, $p < 0.05$) (Fig. 5B). Surprisingly, when the highest dose of Ghr (3 nmol/µl) was used, previous administration of the NOS inhibitor failed to inhibit the Ghr effects upon the threshold to generated LTP (ACSF + Ghr 3 nmol/µl vs. L-NOArg + Ghr 3 nmol/µl = 9 ± 3.3 vs. 9 ± 3.5 Hz, unpaired *t*-tests, $p > 0.05$) (Fig. 5B). Moreover, the inhibitor by itself did not affect the threshold to generate LTP measured 24 h. after inhibitor administration, when compared to ACSF group (ACSF + ACSF vs. L-NOArg + ACSF = 87 ± 9.6 vs.85 ± 5 Hz, unpaired *t*-test, $p > 0.05$) (Fig. 5B).

An interesting result arose from the linear regression analysis between the behavioral and electrophysiological data. This analysis showed a significant correlation ($r^2 = -0.865$) between latency time 24 h after training in the step-down inhibitory avoidance task (as indicator of memory retention) and the threshold to generate LTP in the hippocampal dentate gyrus (Fig. 6).When the high-frequency stimulation (HFS) protocol was used, all groups (ACSF, Ghr 3, Ghr 0.3, L-NOArg, L-NOArg + Ghr 3, and L-NOArg + Ghr 0.3) showed increases greater than 30 % in fEPSP, after HFS application, when compared to basal fEPSP, and with these rises being maintained for at least 60 min (F (4, 88) = 34.51, $p \leq 0.05$). The magnitude of the increases was comparable between groups (F (20, 88) = 1.05, $p > 0.05$) (Fig. 5C).

3. Discussion

The present study showed that intra-hippocampal (CA1) Ghr administration in rats increased the NOS activity, suggesting that Ghr induced activation of the NOS/NO pathway in the hippocampus. These findings are consistent with the localization of the nNOS isoform in hippocampus. It has been demonstrated that nNOS is highly concentrated within the dentate gyrus of the hippocampus [29] and that the perforant pathway stimulation resulted in an increase in the number of nNOS-immunoreactive neurons in the stratum radiatum of the CA1 and CA3 subfields of the hippocampus proper, and the hilus of the dentate gyrus [30].

The results also showed that NOS activity was different in trained and untrained animals. Ghr administration increased NOS activity, but only for the highest dose (3 nmol/µl) in the untrained group, whereas Ghr increased NOS activity in a dose-dependent manner in the trained group. These results could probably be explained by assuming that the training initiate NOS activation while posterior actions of Ghr induce additional increases of intracellular calcium, which stimulate the NOS activity.

Our results using the inhibitor L-NOArg confirm the participation of the NOS/NO pathway in the Ghr induced effects on memory consolidation, since pre-administration of the inhibitor in Ghr treated rats (0.3 nmol/µl) produced inhibition of NOS activity, with short-term and long-term memory performance returning to control levels. When L-NOArg was administered previous the highest dose of Ghr (3 nmol/µl), it only prevented the effect induced by Ghr on short-term memory. This result could probably be attributed to a transient inhibition of NOS activity by the inhibitor at the dose used in this experimental model.

The evidence presented in this paper indicated that the increase of NOS activity and subsequent modifications in NO levels could be one of the endogenous factors in the hippocampus mediating the Ghr effect on memory. This is, as far as we are aware, the first report showing that intra-hippocampal Ghr effects on memory consolidation could be associated with increased NOS activity.

In the present study, we have also demonstrated that in vivo-Ghr administration into the CA1 hippocampus reduced the threshold values to generate LTP in the dentate gyrus (Fig. 5), which is the first synaptic contact of input information arising the hippocampus from entorrinal cortex [31–33]. Our results also showed a significant negative correlation between this electrophysiological phenomenon and the Ghr effect on the inhibitory avoidance task (Fig. 6).

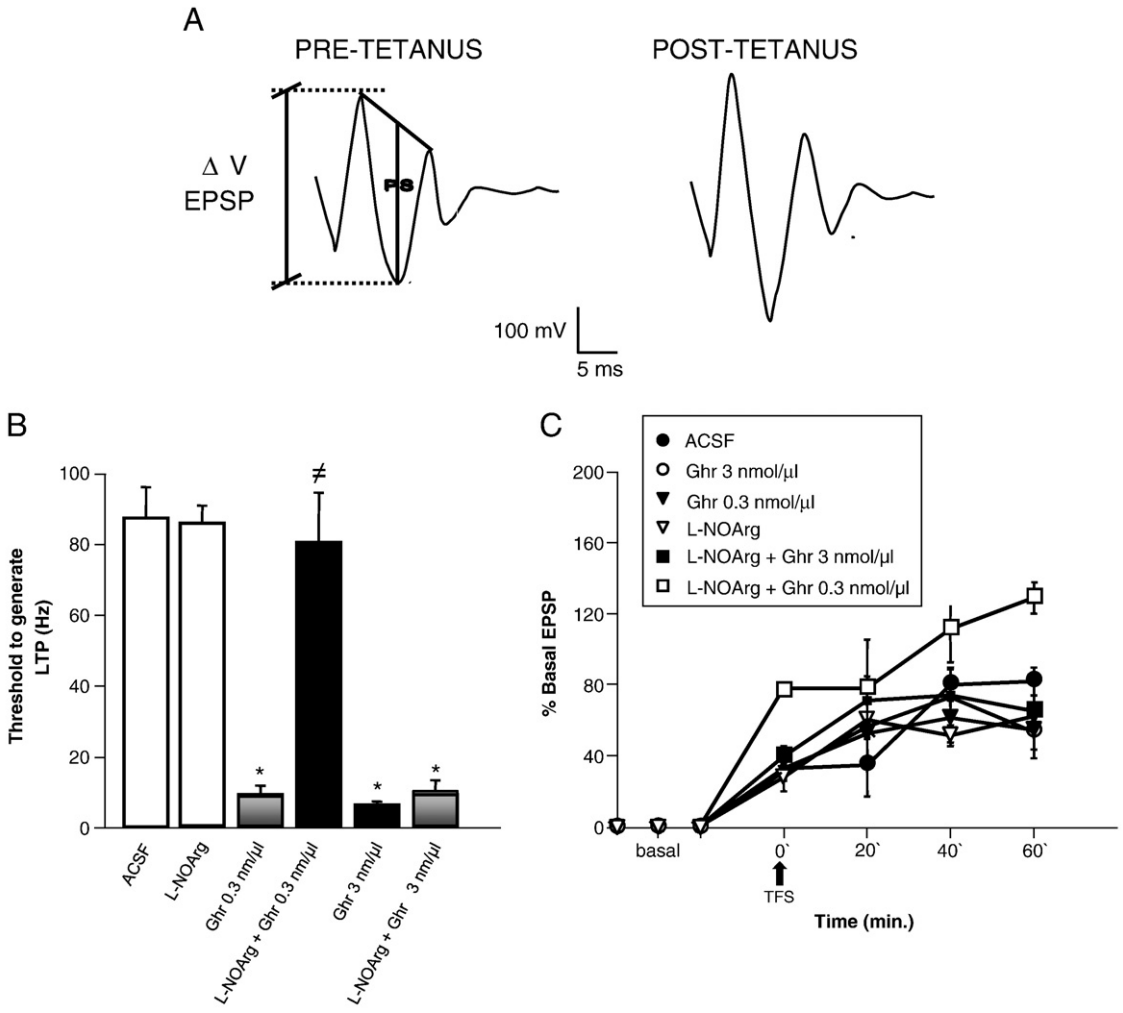

Fig. 5. Inhibition of NOS activity affects the threshold to generate LTP in Ghr-treated animals. Panel A shows an example of a field excitatory post-synaptic potential (fEPSP) trace before an effective tetanus (basal) and after an effective tetanus. Lines indicate the amplitude of fEPSP and Population Spike (mV). LTP was considered to have occurred when one of these parameters increased by at least 30% compared to basal. Panel B shows a bar graph indicating the threshold to generate LTP in Hertz (Hz), in all groups. Bars represent means and lines ± SE. Panel C shows line graph displaying the percentages of the increase with respect to the basal at time 0, 20, 40 and 60 min. after HFS. Each point represents a mean for a specific time, and lines represent ± SEM. *: significant differences related to control animals (ACSF), $p \leq 0.05$. #: significant differences related to Ghr 0.3 nmol/µl, $p \leq 0.05$.

Thus, we can hypothesized in consequence that the hippocampal three-synaptic loop was affected by Ghr, making the post-synapses in the dentate gyrus more sensitive to stimulation by reducing the threshold frequency needed for LTP generation. Diano et al. [27] showed that in vitro-Ghr administration promotes LTP generation in CA1 region of the hippocampus, increasing the EPSP slope. In our experimental model, the EPSP slope in Ghr treated animals was similar to the control animals, this difference could be attributed to the differences in to the region of the hippocampus considered, as well as to differences in the protocol used.

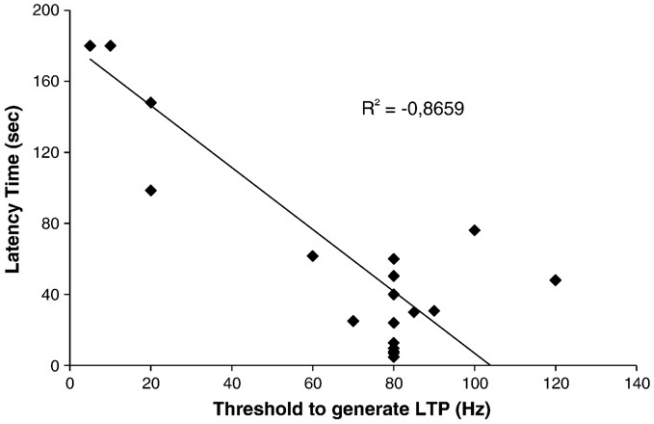

Fig. 6. Correlation between latency time (coordinate) and threshold to generate LTP (abscissa) in hippocampal slices obtained after the step-down test. The spearman rank correlation coefficient was $r_s = -0.865$; $p \leq 0.05$.

Moreover, it has been previously demonstrated that LTP in the hippocampal dentate gyrus, as well as in CA1, requires the activation of NMDA receptors [34], [35], and that the Ca^{2+} influx through NMDA receptors provides the Ca^{2+} signal necessary for the induction and maintenance of LTP signal [36–38]. Consequently, bearing in mind the above-mentioned findings, our results could probably be explained as follows: it is possible that an additive action between the two receptor systems occurs in the hippocampus (NMDA — GHS-R) during the consolidation in step-down test. Thus, the activation of hippocampal GHS-R by Ghr [3,6,7] induces the NOS activation by the additional increases in the Ca^{2+} concentration. The level of NOS activation obtained after ghrelin administration in trained and untrained animals with the same dose supports this hypothesis. Another possibility for explained our results could be that Ghr promotes an indirect NMDA receptor activation, thus stimulating NOS activity through the NR 2b subunit [39]. Further experiments are necessary to clarify these hypotheses.

In conclusion, in our experimental model, the peptide effect on memory consolidation could be related to activation of the NOS/NO signaling pathway and reduction of threshold to generate LTP the dentate gyrus.

4. Materials and methods

4.1. Animals

Male Wistar rats weighing 260–290 g were maintained under controlled temperature at 21 ± 1 °C and a light/dark cycle (12 h light, 12 h dark) with food and water ad libitum. Rats were handled daily for 7 days before the experiments.

All procedures were conducted according to the National Institutes Health (NIH) Guide for the Care and Use of Laboratory Animals (Publications No. 80-23, 1996) and approved by the local Animal Care and Use Committee. Every attempt was made to minimize the number of animals used and their suffering.

4.2. Experimental procedure

4.2.1. First set of experiments: hippocampal NOS activity in Ghr administered animals

These experiments were performed to investigate if Ghr administration in the hippocampus would induce changes in NOS activity.

One group of animals (untrained animals) was administrated with ACSF or three different doses of Ghr (0.03, 0.3 or 3.0 nmol/μl). Another group of animals (trained animals) was placed on the platform in the step down box, and directly after stepping down, the rats received a 2 s of scrambled foot shock (0.4 mA). Immediately after shock animals received ACSF (ACSF-trained) or three different doses of Ghr (0.03, 0.3 or 3.0 nmol/μl) (Ghr-trained). For all groups, 30 min after the Ghr administration the NOS activity was quantified. The time was chosen according to previous data [10] and pilots' experiments in our laboratory (data not shown).

4.2.2. Second set of experiments: effect of NOS inhibition on memory retention and NOS activity in hippocampus of Ghr treated animals

These experiments were designed to evaluate if the changes in NOS activity induced by Ghr in hippocampus contributed to the Ghr-induced increase in memory consolidation. We used the nonselective and reversible NOS inhibitor, L-NOArg. Animals were separated into the following two groups:

a) Behavioral experiments: animals were injected in the hippocampus with ACSF, L-NOArg, Ghr (0.3 or 3.0 nmol/μl) or L-NOArg prior to Ghr administration, immediately after training in the step-down. The latency was measured at 1 or 24 h post training.
b) Determination of the NOS activity: animals were injected in the hippocampus with ACSF, L-NOArg, Ghr (0.3 or 3.0 nmol/μl) or L-NOArg prior to Ghr administration, immediately after training in the step-down test. Thirty minutes later, the animals were sacrificed and the NOS activity was quantified. Different animals were employed for each dose.

4.2.3. Third set of experiments: effect of NOS inhibition in Ghr treated animals on the threshold to generate LTP in the hippocampal dentate gyrus

These experiments were designed to evaluate if the changes in NOS activity induced by Ghr in hippocampus could be correlated with electrophysiological changes. We used the L-NOArg inhibitor. The animals were injected into the hippocampus with ACSF, L-NOArg, Ghr (0.3 or 3.0 nmol/μl) or L-NOArg prior to Ghr administration, immediately after training in the test. The latency was measured 24 h post training and then the animals were sacrificed for the electrophysiological experiments (described elsewhere by Varas et al. [40]).

4.3. Surgery

The animals were anesthetized with 55 mg/kg ketamine HCl and 11 mg/kg xylazine (both Kensol könig, Argentina) and placed in a stereotaxic apparatus. Then, rats were implanted bilaterally into the hippocampus with steel guide cannula, according to the atlas of Paxinos [41]. The coordinates relative to bregma were anterior: −4.3 mm; lateral: ±4.0 mm; vertical: −3.4 mm. Cannulas were fixed to the skull surface with dental acrylic cement. Animals were injected with different drugs using a Hamilton syringe connected by polyethylene tubing to a 30-gauge needle. Each infusion of 0.5 μl per side was delivered over a 1 min period.

4.4. Drugs

The Ghr peptide (Neosystem, France) was resuspended in ACSF to give final concentrations of 0.03, 0.3 and 3.0 nmol/μl.

L-NOArg (Sigma) was resuspended in ACSF to give a final concentration of 2 μg/μl as previously described [42]).

L-NAME (Sigma) was resuspended in Krebs Ringer buffer, to give a final concentration of 100 nM.

[U-^{14}C] arginine (PerkinElmer) packaging 0.1 mCi/ml (3.7 MBq/ml), 360 mCi/mmol, was diluted in buffer krebs Henseleik to obtain a final concentration of 0.28 μmol/ml.

All injections were applied between 10:00 am and noon in order to prevent variations due to circadian rhythms.

4.5. Step-down test (inhibitory avoidance)

Rats were subjected to one trial in the step-down test. The training apparatus was a 50 × 25 × 25 cm plastic box with a 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 to step down by placing the four paws on the grid was measured. In the training session, immediately upon stepping down, the 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 1 or 24 h after training in order to measure short-and long-term memory, respectively, in the different animals group. In both cases test sessions were identical to the training session except that not shock was given. A ceiling of 180 s was imposed on the retention test measures. Latency was taken as a measure of memory retention.

4.6. Histology

After the behavioral test, rats were anesthetized with chloral hydrate, cardially perfused with paraformaldehide (4%) and their brains were removed. Frontal sections were cut in cryostat (Leica), and the injection size was localized. Only results obtained from animals in which the tips of the cannulas were placed into the hippocampus were considered.

4.7. Electrophysiological procedures

Twenty-four hours after the step-down test, electrophysiological experiments were carried out using two different protocols to generate LTP in an *in vitro* hippocampal slice preparation described elsewhere by Pérez et al. [43]. For both protocols, rats were sacrificed between 11.00 am and noon to prevent variations caused by circadian rhythms or nonspecific stressors [44]. The hippocampal formation was dissected, and transverse slices of approximately 400 μm thick were placed in a (BSC-BU Harvard Apparatus) recording chamber, perfused with 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, St. Louis, MO, USA) saturated with 95% O_2 and 5% CO_2. The rate of perfusion was 1.6 ml/min, and the bathing solution temperature was kept at 28 °C by the use of a temperature controller (TC-202A Harvard Apparatus). A stimulating electrode made of two twisted wires, which were insulated except for the cut ends (diameters 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 this study. Field excitatory post synaptic potentials (fEPSP) that responded to 0.2 Hz stimuli were sampled for 20–40 min. Once no further changes were observed in the amplitude of fEPSP or in the amplitude of population spike (PS) one of the stimulation protocols were applied. In the first protocol used the threshold to generate LTP was determined. The PP was primed with a train of pulses (0.5 ms each) of 2 s duration, of increasing variable frequency. They were delivered to the PP by an A310 Accupulser Pulse Generator (World Precision Instruments Inc.), at intervals that ranged from 10 to 20 min, starting with 5 Hz tetanus, and followed by 10, 25, 50, 75, 100, 150 and 200 Hz. Ten minutes after each tetanus a new averaged response was recorded. If LTP was not observed, a new stimulation at the next higher frequency was applied. LTP was considered to have occurred when the amplitude of the fEPSP or the amplitude of the PS recorded after the tetanus, had risen by at least 30% and persisted for 60 min. Once LTP was achieved, no further tetanus was given. For each animal, another hippocampal slice was used to apply the second protocol to induce LTP, using a tetanization paradigm consisting of three 100 Hz HFS trains (each of 1 s duration) given at 20-s intervals. LTP was considered to have occurred as it was described above for the first protocol. All collected data were recorded and stored for future analysis.

4.8. Determination of nitric oxide

Animals were killed by decapitation. Brains were removed and the hippocampus was dissected to measure total NOS activity, using L-[U-^{14}C] arginine as described by Bredt et al. [29]. Briefly, each rat entire hippocampus was placed in a tube with 0.5 ml Krebs–Ringer bicarbonate (KRB) and incubated in a shaker at 37 °C in 95% O_2 5% CO_2 for 8 min in order to stabilize the tissue. Then, tissue was homogenized using a manual homogenizer, in 500 μl of Normal Buffer [20 mM Hepes (pH 7.4), 1 mM dithiothreitol (DTT), 0.45 mM $CaCl_2$ and 400 μM NADPH] or in free Ca^{++} buffer (Hepes 20 mM, DTT 100 mM, EGTA 2 mM, EGTA 0.2 mM, NADPH 400 μM), and homogenate aliquots were reserved for protein determinations [45]. Homogenate aliquots of 200 μl were incubated with 0.1 μCi L-[U-^{14}C] arginine for 30 min in a shaker at 37 °C in 95% O_2 5% CO_2. Samples were centrifuged at 10,000 rpm at 4 °C for 10 min. Supernatant aliquots (400 μl) were eluded in individual columns of Dowex AG 50 W-X8 200–400-mesh sodium form (Fluka), previously stabilized with 20 mM Hepes (pH 7.4) in order to separate [^{14}C] citrulline from the supernatants. They were then washed with 3 ml double distilled water, and the [^{14}C] citrulline in the eluent was quantified by liquid scintillation using a β-counter.

Due to the fact that L-citrulline and NO are generated in equimolar amounts, and since L-citrulline is stable whereas NO is not, quantification of L-citrulline is an indirect measurement of NO production, thereby indicating NOS activity [46].

The data were expressed as pmol of NO generated/mg of protein.

An additional determination was made by adding N (G)-nitro-L-arginine methyl ester (L-NAME) (100 nM), an inhibitor of NOS activity, to the hippocampus homogenate for all groups. Under this condition, the activity of the enzyme was completely inhibited (data not shown).

4.9. Statistics

The data from NOS activity determination and electrophysiological experiments were expressed as means ± SEM and analyzed by analysis of variance (ANOVA) and an unpaired *t*-test, respectively. The data from NOS activity determinations and the first set of experiments were evaluated with a one-way ANOVA. The data from NOS activity determinations in the second set of experiments together with the data from electrophysiological experiments were evaluated with a two-way ANOVA. For each ANOVA an LSD post hoc test was applied to determine the significant source detected with p values ≤ 0.05 being considered statistically significant. Since the variables being analyzed from step-down inhibitory test do not follow a normal distribution and its variance does not fulfil the assumption of homoscedasticity, these were expressed as medians (inter-quartile range) and analyzed by non-parametric tests (Mann–Whitney or Kruskal–Wallis) followed by Dunn's post hoc comparisons. Values $p \leq 0.05$ were accepted as having statistical significance.

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