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Ghrelin Is Effective on Passive Avoidance Memory by Altering the Expression of *NMDAR* and *HTR1a* Genes in the Hippocampus of Male Wistar Rats

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Abstract

Background: Memory-dependent psychological behaviors have an important role in life. Memory strengthening in adulthood to prevent its defects in aging is a significant issue. The ghrelin endogenous hormone improves memory by targeting glutamatergic and serotonergic circuits. Also, citicoline, a memory strengthening drug in aging, is not recommended to adults due to its side effects. The current study aims to test that ghrelin treatment, like citicoline, would improve passive avoidance memory via expression of the genes encoding the N-methyl-D-aspartate receptor (*NMDAR1)* and the serotonin receptor 1A (*HTR1a)* involved in this process.

Methods: Five groups of adult male rats received (1) saline (as control), (2) 0.5 mg/kg citicoline, or (3-5) 0.3, 1.5, and 3 nmol/µl ghrelin). The rats received the drugs via intra-hippocampal injection. Passive avoidance memory was determined using a shuttle box device. The latency to enter the dark chamber before (IL) and after (RL) injection and the total duration of the animal's presence in the light compartment (TLC) were evaluated. Then, the gene expression rates of *NMDAR1* and *HTR1a* were measured by the Real-Time PCR.

Results: Ghrelin and citicoline had some similar and significant effects on passive avoidance memory, and both increased *NMDAR1* and decreased *HTR1a* expression.

Conclusions: Ghrelin, like citicoline, improves passive avoidance learning by altering the *NMDAR1* and *HTR1a* expression in the hippocampus.

Keywords: Citicoline, Ghrelin, *HTR1a*, Intrahippocampal injection, *NMDAR1*, Passive Avoidance Memory.

Introduction

Memory-dependent psychological behaviors have an important role in human life. Currently, the increasing prevalence of memory disorders has become a worry (1, 2). Thus, researchers are constantly looking for more effective drugs to improve memory than those currently in use. Research has shown that ghrelin plays a role in improving memory. This hormone is produced by the stomach and some other tissues, and once activated, affects target tissues (acyl-ghrelin). The presence of ghrelin receptors, also known as growth hormone secretagogue receptors (GHSRs) and members of the G-protein coupled receptor family, in the hippocampus indicates its role in memory. In the hippocampus, the main ghrelin receptor is GHSR1a. The functional mechanism of ghrelin is not yet fully understood. Because this 28-amino acid peptide targets glutamatergic and serotonergic circuits, ambiguities of its mechanism may be related to these pathways and their gene expression (3-5).

Also, citicoline diphosphate-choline (CDP-choline) or citicoline, an intermediate product in phosphatidylcholine production (6) improves passive avoidance memory, cognitive levels, and hippocampal-dependent memory impairment (7,

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8). It is the current drug for the improvement of memory disorders in aging (8), but is not recommended to adults for the prevention of memory disorders, due to possible adverse side effects.

The N-methyl-D-aspartate receptor, a glutamate receptor and ion channel protein in the central nervous system, encoded by the *NMDAR1* is important for controlling synaptic plasticity, memory function, and long-term potentiation (LTP) in the hippocampus (9, 10). Also, the serotonin 1a receptor encoded by the *HTR1a* is an autoreceptor, and coupled with G-protein (Gi), the most widespread of the serotonin (5-hydroxytryptamine, 5-HT) receptors in the hippocampus, is related to memory disorders (3, 11, 12).

This study attempts to compare the mechanism of action of ghrelin and citicoline through behavioral tests and the expression of related genes. In this study, citicoline and acyl-ghrelin were injected into the hippocampus, then passive avoidance memory was analyzed by the shuttle box apparatus. Next, *NMDAR1* and *HTR1a* expression was evaluated in the hippocampus.

Materials and Methods

Animals

Sigma-Aldrich Chemicals (St. Louis, MO) company was the source of purchase of Berberine hydrochloride, pilocarpine hydrochloride, scopolamine methylnitrate & diazepam and all the other chemicals were used.

Animals

We obtained 30 adults male Wistar rats (aged 8 weeks, 230–250 g) from the Neuroscience Research Center of Shahid Beheshti University. They were kept in a controlled room with a 12 h light/dark cycle with lights on at 7:00 a.m. and temperature of 22±2 °C. Food and water were available. All empirical steps including maintenance, stereotaxic surgery, intra-hippocampal injection, shuttle box testing, euthanizing, and hippocampal extraction were confirmed by the Regional Ethics Committee of Shahid Beheshti University (IR.SBU.REC.1399.047).

Experimental groups

The rats were randomly divided into five groups of six rats each. The groups were (1) control, (2) 0.5 mg/kg citicoline, and (3-5) 0.3, 1.5, or 3 nmol/µl ghrelin. Drug doses were selected based on previous research (13, 14). Ghrelin was purchased from Alfa Aesar Company, Germany, and Citicoline was purchased from Qilu Pharmaceutical Company, China.

Stereotaxic surgery

The surgical method was based on previous research (15). First, animals were anesthetized with an intraperitoneal injection of a mix of xylazine-ketamine (12 and 60 mg/kg, respectively, both from Kensol Konig, Argentina). The hippocampus was bilaterally cannulated. The animals were situated on a stereotaxic framework (Stoelting Co, Illinois, USA) to implant a 22-gauge stainless steel guide cannula into the CA1 (dorsal hippocampus) with coordinates of "AP= -3.8, L= ±2.2 and DV= -2.7" (16). The guide cannula was anchored to the skull by three stainless steel screws and dentistry acrylic cement. It was shorter than the injection cannula (0.5 mm). After surgery, animals were returned to solo cages to make a recovery for one week. One week after surgery, saline, 0.5 mg/kg citicoline, or 0.3, 1.5, or 3 nmol ghrelin were bilaterally injected into CA1 in a volume of 1 µl using a 27-gauge stainless steel injector connected to 1 µl Hamilton microinjection system by 20-polyethylene tubing during 1 min at 9-10 a.m. The control group received only saline. Injection materials were dissolved in sterile 0.9% saline.

Shuttle box apparatus test

An ST-5500 shuttle box apparatus (Borj Sanat Co. Tehran-Iran) was used to determine the effects of citicoline and ghrelin on passive avoidance memory based on previous studies protocols (17, 18). The shuttle box is a standard device for training trials of passive avoidance memory. One week after surgery, rats were allowed to habituate in the test room for at least half an hour, then for the habituation step, each rat was situated in the light chamber of the

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device. Ten s later, the guillotine door, between the bright and dark chamber, was opened and the rat was allowed to enter the dark chamber. Then the guillotine door was closed, and 30 s later, the animal was returned into its cage. After 30 min, the training step was started; the rats were trained as in the habituation step except that as soon as the door was closed, a shock was delivered to the floor (50 Hz, 3 s, and 1 mA intensity). The initial latency (IL) time to enter the dark chamber was recorded. Animals with ILs> 100 s were excluded from the study. The animal was removed from the device and after two minutes, was retrained. This was repeated several times for at least 120 s; the animal did not enter the dark chamber and remained in the bright room. After this step, the animal received the drug as described. Post-training injection aimed to measure the effects of the drug on the memory formation process. The final test step was performed 24 h after the training. Throughout this step, the animal was put in the light chamber and after 10 s the guillotine door was opened. After entering all four legs into the dark chamber, the retention latency (RL) was recorded for each rat. Also, the time the animal stayed in the light compartment (TLC) was calculated at 300 s. During the testing step, no electric shock was given.

Real-Time PCR

After the last test, the animals were euthanized under stress-free conditions by beheading. The brain was removed and the hippocampus collected (19, 20). The hippocampus was frozen in liquid nitrogen and stored at -80 °C until the real-time measurement. RNA was extracted using Trizol reagent according to the manufacturer's instruction (GeneAll Biotechnology, Korea). The purity and quantity of the RNA were determined using Nanodrop (Thermo Scientific, Germany). High-quality RNAs (A260/280≥1.8-2.2) were chosen and kept at -80 °C to be used for cDNA synthesis later. Then RNA was transcribed into cDNA using a reverse transcription kit (Takara, Bio, Inc). To determine the cDNA's integrity, PCR was performed using glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene for normalizing data. The gene sequences were extracted from the gene bank to design of primers using the "Primer3" site. Primer sequences selected for this study are listed in Table 1. The reaction was performed at 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and then 72 °C for 30 s. Melting curves were analyzed according to the dissociation stage data and reactions were selected with a single peak at melting temperature for further analysis. Eventually, data was normalized with GAPDH, and the $2^{-\Delta\Delta Ct}$ technique was used to compare the changes in gene expression between the experimental and control groups (fold change) (21, 22).

Table 1. Primers used for this study.

Gene	Primer sequence (5′–3′)	NCBI accession number	Product size (bp)
NMDAR1	F: GGAGGAAGATGCACTGACCC R: GTCATTGATGCCCGTGATGC	XM_006233556.3	296
HTR1a	F: CTCTCATTTTCTGCGCGGTG R: TGCAGCACAGTACATCCAGG	NM_012585.1	228
GAPDH	F: CGGCCTTCCTCATTCTTAGCTT R: ACGGAAACCCTGCCATCCAT	NM_017008	103

NMDAR1: N-methyl-D-aspartate receptor subunit 1; *HTR1a*: 5-Hydroxytryptamine receptor 1A; *GAPDH*: Glyceraldehyde 3-phosphate dehydrogenase; F: Forward; R: Reverse.

Statistical analysis

First, data normality was assessed by the Shapiro-Wilk test. The shuttle box test data were presented as medians (min, max). To assess differences between the groups, ANOVA and Tukey's test was used, and statistics were analyzed using GraphPad Prism, version 9 software. P values< 0.05 were considered to be statistically significant.

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For real-time data analysis, gene Ct values were normalized with the reference gene, and then the ΔΔCt method was used to compare gene expression between the groups. Also, the Tukey test was used to compare groups two by two. The ANOVA test was used to determine whether gene expression differences between groups were statistically significant. These calculations were performed by Genex 6 software, statistical analysis by GraphPad Prism, version 9 software. P values< 0.05 considered to be statistically significant.

Results

Effect of ghrelin on shuttle box test

The duration delay time of the entering the dark chamber, before (IL) and after (RL) injection, as well as, the total duration of the presence in the light compartment (TLC) were evaluated and compared following the Shuttle Box Test (Fig. 1). No significant differences in terms of IL parameters were seen between the groups (Fig. 1A). RL and TLC were significantly greater in all treated groups than in controls (Figs. 1B and 1C, respectively).

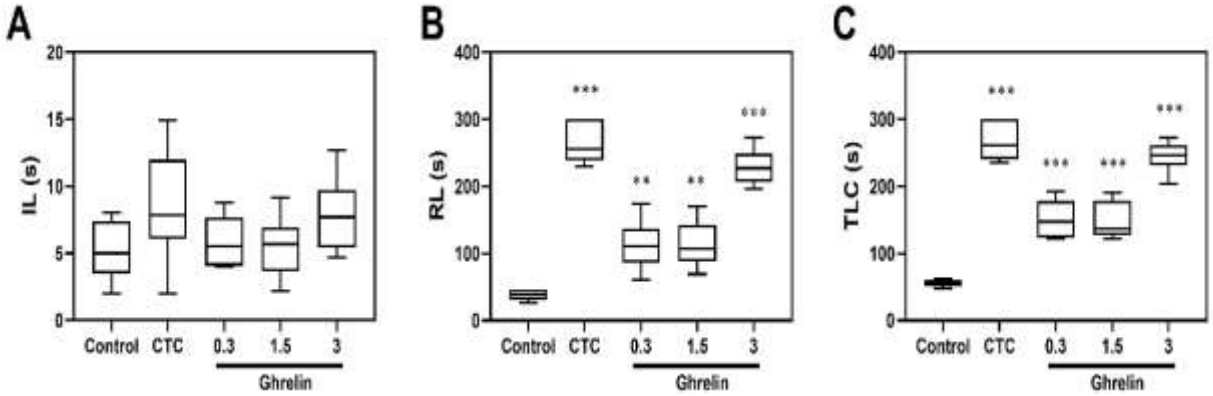

Fig. 1. Shuttle box test results. Shows the latency time (in seconds, s) to enter the dark chamber before foot shock (IL, A), retention phase (RL, B), and time in the light compartment (TLC, C). For IL (A), no significant differences were seen between the control and experimental groups. RL (B) and TLC (C) were both significantly greater than control for both the citicoline- and 0.3, 1.5, and 3 nmol ghrelin-treated groups (**p< 0.01, ***p< 0.001, vs. control group). Data are shown as median (min, max) of six animals per group. Abbreviation: CTC, citicoline; IL, initial latency; RL, retention latency; TLC, time in the light compartment.

Effect of ghrelin on NMDAR1 gene expression

NMDAR1 expression was significantly greater in the right hippocampus than in controls following treatment with CTC or 3 nmol ghrelin, and in the left hippocampus following treatment with CTC only. *NMDAR1* expression was significantly greater in the right than in the left hippocampus groups treated with CTC or 3 nmol ghrelin (Fig. 2).

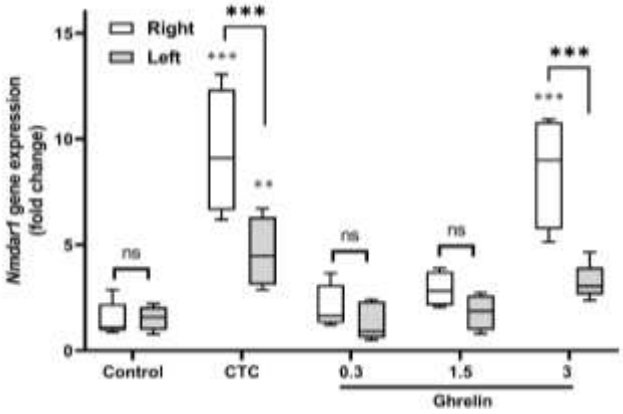

Fig. 2. *NMDAR1* gene expression between groups in the right and left hippocampal regions. Data are shown as median (min, max) of six animals per group. *NMDAR1* expression was significantly greater in the right hippocampus than in controls following treatment with CTC or 3 nmol ghrelin, and in the left hippocampus following treatment with CTC only (**p< 0.01, ***p< 0.001, vs. control group). Comparison between the right and left hippocampus in each group shows the significant difference at p< 0.001 between CTC and 3 nmol ghrelin. Abbreviation: CTC, citicoline; *NMDAR1*, N-methyl-D-aspartate receptor subunit 1; ns, not significant.

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Effect of ghrelin on HTR1a gene expression

HTR1a expression was significantly less in both the right and left hippocampus than in controls following treatment with CTC or 3 nmol ghrelin. *HTR1a* expression did not differ significantly between the right and left hippocampus for the controls or any of the treatment groups (Fig. 3).

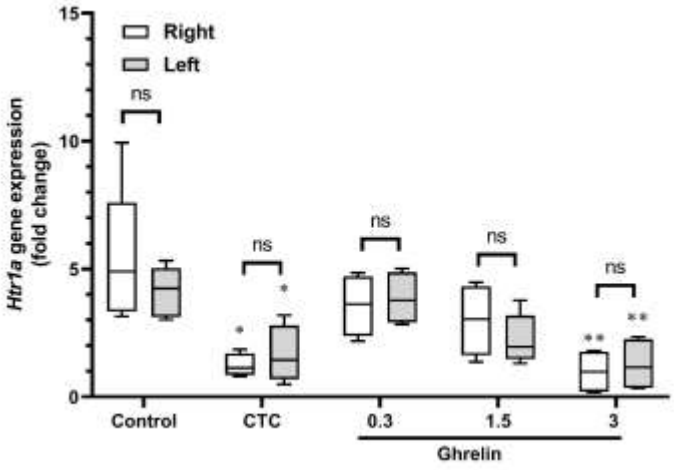

Fig. 3. *HTR1a* gene expression between groups in the right and left hippocampal regions. Data are shown as median (min, max) of six animals per group. *HTR1a* expression was significantly greater in the right hippocampus than in controls following treatment with CTC or 3 nmol ghrelin, and in the left hippocampus following treatment with CTC or 3 nmol ghrelin (*p< 0.05, **p< 0.01, vs. control group). The comparing of changes in *HTR1a* gene expression between the right and left hippocampus in each group shows no significant difference. Abbreviation: CTC, citicoline; *HTR1a*, 5-Hydroxytryptamine receptor 1A; ns, not significant.

Discussion

Our results showed that citicoline and 3 nmol ghrelin increased both RL and TLC mean times 24 h after the injection, indicating improved passive avoidance memory. This finding agrees with the work of Carlini et al. (13, 15), which showed that ghrelin improves passive avoidance memory. Also, they stated that long-term memory can be improved by ghrelin. These studies confirm and justify our results, but until now, the effects of ghrelin and CTC had not been compared simultaneously. Although the rate of passive avoidance memory improvement in the ghrelin-treated group (3 nmol) was significantly greater than that of the controls, it was less than that of the citicoline-treated group. This discrepancy is probably due to the differences in their mechanisms of action. Ghrelin is a peptide that affects target cells via its receptors (3), but citicoline is a known cellular substance that easily and quickly enter the cell (6). Despite this difference, the effect of ghrelin is similar to that of citicoline.

We also showed that *NMDAR1* expression increased significantly after ghrelin injection (3 nmol), similar to citicoline. Studies have shown that ghrelin, via GHSR1a, stimulates NMDA receptor-mediated synaptic transmission by phosphorylation of this receptor in the hippocampus (23, 24). In this regard, Secades reported that citicoline significantly increases ATP brain levels, which positively effect secretion and increase glutamate in the synaptic space (25). This event, in turn, increases NMDA receptor expression, which improves memory via increased synaptic plasticity (26). These studies confirm our results, although, until now, the effects of these two drugs had not been compared simultaneously.

Both citicoline and three nmol ghrelin inhibited *HTR1a* expression. According to studies in rodents, stimulation of 5-HT1A receptors generally produces memory impairments. In contrast, *HTR1a* antagonists facilitate certain types of memory (27). Strong

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evidence indicates that memory impairment is related to both post-synaptic *HTR1a* stimulation and high doses of *HTR1a* (28). Hansson et al. (2011) reported that ghrelin inhibits the serotonergic system. These findings confirm our results. Until now, the effect of citicoline on *HTR1a* had not been studied.

In addition, we found that *NMDAR1 and HTR1a* expression differed significantly between the right and left hippocampus in homogeneous groups. Consistent with our results, even without treatment, the right and left areas differ functionally (29, 30), however, this difference was significantly exacerbated by citicoline and ghrelin.

Summarizing the results of the present and previous studies, ghrelin significantly improved passive avoidance memory while increasing *NMDAR1* and decreasing *HTR1a* expression. Also, we found that citicoline and ghrelin had similar and significant effects on the hippocampus. Ghrelin has fewer side effects than citicoline, but it appears that after further studies, ghrelin may be used in some cases, including drug interactions, side effects, and allergies, to improve passive avoidance memory. Additional medical and behavioral studies are needed to confirm our results.

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