Central Injection of Ghrelin Improves Motor Balance in the Rotarod Test in the Rats: Altering the Expression of *Drd1* Gene

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Abstract

Introduction: Motor learning consolidates in adulthood, and its defects begin to appear with aging. Ghrelin, an endogenous peptide, improves memory and learning, targeting dopaminergic circuits. While cytidine diphosphate choline (citicoline) is known as a common drug for enhancing memory and learning in aging, it is not recommended for adults due to its side effects. The current study aimed at investigating if ghrelin treatment would improve motor learning via the expression of a relevant gene.

Methods: For this experimental study, adult male Wistar rats were randomly divided into five groups: control group, three groups of ghrelin treatment (0.3, 1.5, and 3 nmol/ μ L), and one group with citicoline treatment. The injections were done intra-hippocampally. The motor learning rate was determined using the rotarod performance test by measuring the resistance to falling. Then the expression of dopamine receptor type D1 (*Drd1*) gene in the hippocampus was measured by a real-time polymerase chain reaction (PCR).

Results: Ghrelin (3 nmol/ μ L) and citicoline had similar and significant effects on motor learning improvement ($P < 0.01$). Both drugs significantly increased *Drd1* gene expression ($P < 0.001$).

Conclusion: Ghrelin, like citicoline, improves motor learning by altering the expression of *Drd1* gene in the hippocampus.

Keywords: Ghrelin, Cytidine diphosphate choline, Rotarod performance test, Real-time polymerase chain reaction

Introduction

Nowadays, as an attempt to understand the changes that occur with aging, numerous researchers in advanced nations have been doing extensive research in the phenomenon.¹ One area of the changes associated with aging is the decline in the learning of motor skills, such as the ones which require balance.^{2,3} A core risk factor in falling is the loss of postural stability.⁴ Even though most falls do not bring about hazardous injuries, they will somehow affect one's competency⁵ and quality of life.⁶ Thus, researchers are always looking for ways to improve motor learning and maintaining balance.

Ghrelin is a brain-gut peptide that is active in its acylated state.^{7,8} Ghrelin receptors (GHSRs; growth hormone secretagogue receptors) act through G-protein receptors.⁷ GHSRs are expressed

in various tissues such as the hippocampus, indicating their involvement in learning. GHSR1a is identified to be the main receptor of ghrelin in the CA1 region of the hippocampus.⁹⁻¹¹ Since this 28-amino acid peptide⁷ targets dopaminergic circuits,^{10,12} ambiguities of its mechanism in learning may be related to these pathways and the expression of their genes.

On the other hand, cytidine diphosphate choline (citicoline) is known to be a memory and learning enhancer in aging, and brain degenerative diseases.¹³⁻¹⁵ An intermediate product, cytidine diphosphate-choline generates phosphatidylcholine from choline.¹⁶ Citicoline appears to stimulate cell membrane stabilization by phospholipid biosynthesis; as a result, it boosts memory and learning.¹⁵

Dopamine receptor type D1 (DRD1) is the most abundant dopamine receptor

in the hippocampus.¹⁷ It has become crystal clear that treatments targeting dopamine D1 receptors may improve motor sequence learning.¹⁸

The rotarod performance test is a training approach that has been recurrently demonstrated to boost motor skills' learning and performance, such as balance skills. It has been indicated that a new motor skill is acquired during rotarod training.^{19,20} In this study, we injected ghrelin and citicoline into the hippocampus and used (before and after injections) the rotarod test to compare their effects on motor learning. Moreover, the effect of these substances on the expression of the *Drd1* gene in the hippocampus was investigated.

Materials and Methods

Animals

The Regional Neuroscience Research Center of Iran provided the required 8-week-old adult male Wistar rats, weighing 230-250 g. These rodents were maintained at a controlled room with a specified temperature of $22 \pm 2^\circ\text{C}$. They were kept under 12 hours light/dark cycle with lights turning on at seven in the morning. They had unlimited access to feeding.

Experimental Groups

The animals were categorized randomly into five groups: control, different doses of ghrelin (0.3, 1.5, or 3 nmol/ μL), and citicoline (0.5 mg/kg) (6 rats in each group). These doses were selected based on previous research.^{21,22} Ghrelin was purchased from Alfa Aesar Company, Germany, and Citicoline was bought from Qilu Pharmaceutical Company, China.

Stereotaxic Surgery

The surgical method was implemented according to previous research.²¹ Prior to the operation, a combination of ketamine-xylazine (respectively 60-12 mg/kg, both Kensol Konig, Argentina) was injected intraperitoneally for anesthetizing the rats. Then, the hippocampus was bilaterally cannulated. In this regard, the animals were situated on a stereotaxic framework (Stoelting Co, Illinois, USA) to implant the 22-gauge stainless steel guide cannula into the dorsal hippocampus (CA1) with coordinates of "AP = -3.8, L = ± 2.2 and DV = -2.7" according to the coordinates given in the atlas.²³ The injection cannula was longer than the guide one (0.5 mm). The latter was anchored to the skull by three stainless steel screws and dentistry acrylic cement. After the surgery, for one week the rats were returned to single cages for recovery.

Microinjection

One week after the surgery and health recovery, saline, ghrelin (at doses of 0.3, 1.5, and 3 nmol/ μL), or citicoline were injected bilaterally into CA1 at a volume of 1 μL for 2 minutes with the use of a 27-gauge stainless steel

injector connected to 1 μL Hamilton microinjection by 20-polyethylene tubing (at 9-10 AM). For minimizing solution's backflow, the needle remained in the place for another 2 minutes. The control group received saline. The materials were solved in sterile 0.9% saline.²⁴

Motor Skill Learning Test With the Rotarod Device

The test of rotarod is a standard test of motor performance. Accelerated rotarod training can be deemed to be a reliable paradigm for motor skill learning. It is indicated that when doing rotarod training, the subjects would learn a new motor skill.^{19,20} For this purpose, for probing the effects of ghrelin/citicoline on motor learning, a rotarod device (Borj Sanat Azma Co, Tehran, Iran) armed with a seven cm diameter rod, acceleration capability, automatic timers, and falling sensors was used. Constant acceleration of 4 cm/s^2 was performed until the animal fell from the rod. By adding this constant acceleration, animals had to adapt themselves continuously to the changing velocity. For improving the performance, this intensity was kept both high enough and, yet not too low to stave off fatigue.

In this research, one week after the surgery, at time points 30 min before the injections, 30 and 60 minutes, 3, 6, 12, and 24 hours after the injections, first, the animal was habituated in the test room for at least 30 minutes, and then on the stationary rod of the device for 1 minute. The rats would go under three rotarod trials for each session. The mean time of animal gaiting on the device (the resistance to falling) was recorded by automatic timers. The time interval between the trials was 30 seconds. It is noteworthy that before starting the tests, the walking abilities of the animals in the rotarod performance were evaluated. Accordingly, they were initially put on the device, and those that immediately fell were removed from the experiment.

Real-Time Polymerase Chain Reaction

After the latest rotarod test, the animals were deeply anesthetized with a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg), and then, they were sacrificed under stress-free conditions. After that, the brain was rapidly pulled out, and the hippocampi were cut and removed.²⁵ This long vessel, after a rapid and sudden freeze at the N-tank, was transferred to the Freezer -80 until the real-time measurement. Then, using *TRIzol* reagent, we extracted RNA in accordance with manufacturer's instruction (GeneAll Biotechnology, Korea). Nanodrop (Thermo Scientific, Germany) was adopted for determining the quantity and purity of the extracted RNA. RNases were selected and maintained at -80°C to be used for the synthesis of complementary DNA (cDNA). The concentration of the sample was evaluated and RNA was transcribed into cDNA adopting a reverse transcription kit (Takara, Bio, Inc). For measuring the cDNA integrity, a PCR experience was carried out adopting

glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene for data normalization. For the design of the primers, the DNA sequence of the genes was derived from the gene bank and after that was designed on the site of primer 3. The selected primer sequences of the present study are displayed in Table 1. The reaction was performed at 95°C for 15 minutes, followed by 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and then 72°C for 30 seconds. The analysis of melting curve was performed based upon the data of dissociation stage and the reactions were selected with a single peak at melting temperature for further scrutiny. Finally, data were normalized with GAPDH and the $2^{-\Delta\Delta C_t}$ technique was exerted to compare fold changes in *Drd1* expression between the experimental and control groups.^{26,27}

Statistical Analysis

Firstly, the normality of data was assessed by the Shapiro-Wilk test. The rotarod test results were presented as mean $\pm$ SE. Two-way ANOVA and Tukey's post hoc test were utilized for assessing the differences between the experimental groups using GraphPad Prism software, version 9. A *P* value of < 0.05 was deemed statistically significant.

Moreover, the post hoc test of Tukey was used for binary comparisons among the groups. ANOVA was used to calculate differences in gene expression among the groups. Gene expression differences were calculated using GraphPad Prism software, version 9.

Results

Effect of Ghrelin on the Rotarod Test

Rotarod test results are shown in Figure 1. The mean time of animal gaiting on the device (the resistance to falling) was calculated and compared in different groups at before (0 min) and after (30, 60 minutes, 3, 6, 12, and 24 hours) the injection. There was significant increase after the injection of ghrelin (3 nmol/ μ L) (at 12 and 24 hours; *P*=0.006 and *P*=0.001, respectively) or citicoline (at 12 hours; *P*=0.001) in comparison with control at the corresponding time.

Effect of Ghrelin on *Drd1* Gene Expression

Real-time PCR results have been shown in Figure 2, and Figure 3 demonstrates the analysis of changes in gene expression between the groups in the left and right

regions of the hippocampus. It reports a significant increase in *Drd1* gene expression after the injection of ghrelin 0.3, 1.5, and 3 nmol (*P*=0.817, *P*=0.010 and *P*<0.001 at right; *P*=0.042, *P*=0.070, and *P*<0.001 at left hippocampus, respectively) or citicoline (*P*<0.001 at right; *P*<0.001 at left hippocampus) in comparison with the control group at the corresponding region (Figure 2A, and 2B). Furthermore, the analysis of changes in the gene expression between the groups in the left or right hippocampus showed a significant difference only in the ghrelin 0.3 nmol group (*P*=0.039) (Figure 3).

Discussion

In the present study, for the first time, the impacts of the intra-hippocampal injection of ghrelin or citicoline on motor learning and gene expression of *Drd1* were examined. Moreover, a comparison of the effects of these drugs was performed for the first time. This comparison was done by the rotarod device test and then the real-time test. The results revealed that the injection of ghrelin (3 nmol/ μ L), like citicoline, significantly increased the meantime of rat walking (resistance to fall) on the rotarod device. In this context, Suda et al, state that the injection of ghrelin into substantia nigra (SN) improves motor coordination and latency to fall on the rotarod device.¹² Another study on rotarod documented that ghrelin increases motivation in the motor task.²⁸ As the literature reveals, previous research is consistent with the results of the present study. Research has also been done on the positive effects of citicoline on motor behavior and learning. Citicoline has been shown to improve motor behavioral performance in the rotarod device.²⁹ Bruhwylter et al also suggested that citicoline can have facilitating effects on learning and motor performance.³⁰ This drug increases learning capacity and reduces motor deficits.³¹ As can be seen, previous research confirms the results of this research. It should also be noted that the results of this study revealed that the mentioned effects were time-dependent. Motor learning after ghrelin injection for up to 24 h significantly improved.³² Moreover, significantly increased improvement was observed for motor learning after citicoline injection for up to 24 hours.³¹ Another particular finding of the present study was that although the rate of motor learning improvement in the ghrelin-treated group (3 nmol/ μ L) was significantly higher than that of the control group, it was akin to that of

Table 1. Primers Adopted in the Present Study

Gene	Primer sequence (5'–3')	NCBI Accession Number	Product Size (bp)
<i>Drd1</i>	F:GCTGGCTCCCTTTCTTCATC R:CACCCAAACCACAAACAC	NM_012546.2	111
<i>Gapdh</i>	F:CGGCCTTCCTCATTCTTAGCTT R:ACGGAAACCTGCCATCCAT	NM_017008	103

Note. *Drd1*: Dopamine receptor D1; *Gapdh*: Glyceraldehyde 3-phosphate dehydrogenase; F: Forward; R: Reverse.

Figure 1. The Comparison of Rotarod Test Results at Different Times After Injection of Drugs. Data are exhibited as mean $\pm$ SE of six animals per group. ** Indicate a significance difference at $P < 0.01$ compared with the control group at corresponding time. Abbreviation: CTC, citicoline.

Figure 2. Analysis of Changes of *Drd1* Gene Expression Between Groups in the Right (A) and Left (B) Hippocampal Regions. Data are shown as median (min, max) of six animals per group. Different small letters on boxes indicate a significant difference at $P < 0.05$. Abbreviation: CTC, citicoline; *Drd1*, Dopamine receptor D1.

Figure 3. The Comparison of Changes in *Drd1* Gene Expression Between the Right and Left Hippocampus in Each Group. Data are shown as median (min, max) of six animals per group. * indicates a significant difference at $P < 0.05$. Abbreviation: CTC, citicoline; *Drd1*, Dopamine receptor D1.

the citicoline-treated group. While ghrelin is a peptide that affects target cells by its receptors,³³ citicoline is a known cellular compound that mediates the production of phosphatidylcholine and can easily enter the cell.¹⁶ Needless to say, the effects of ghrelin are similar to those of citicoline in this region.

To further understand the phenomenon of motor

learning, the expression levels of *Drd1* gene were evaluated in the hippocampus via real-time PCR. This study found that *Drd1* gene expression was significantly increased after the injection of ghrelin (3 nmol/ μ L). Previous studies have signalled that ghrelin has a positive interaction with dopamine signaling.¹⁰ Following GHSR1a activation, a physiological relationship is initiated in the simultaneous expression of *Drd1* and GHSR1a through the mechanism of GHSR1a/DRD1 heterodimer formation.³¹ Furthermore, the functional role of DRD1 in the processes of synaptic plasticity in the hippocampus via the ghrelin receptor has been demonstrated to improve memory and learning.¹⁷ These findings can justify the increase in *Drd1* expression following the injection of ghrelin in our study. Moreover, the present study also signifies that *Drd1* gene expression was significantly increased after the injection of citicoline in the hippocampus. Citicoline has been shown to increase the density of dopamine receptor and thereby enhance its availability.³¹ A review of previous studies confirms this particular finding, too. Generally, treatments targeting dopamine D1 receptors can improve motor sequence learning.¹⁸

Conclusion

In conclusion, the present study was the first research examining the impacts of the intra-hippocampal injection of ghrelin or citicoline, on motor learning and gene expression of *Drd1*. Furthermore, a comparison of the effects of these drugs was performed for the first time in this study. The results unveiled that ghrelin and citicoline had similar and significant effects on the hippocampus. These drugs, as was shown, are remarkably effective in improving the rotarod test and motor learning via increasing *Drd1* gene expression. Nevertheless, the effects of ghrelin were less significant than those of citicoline; that said, it seems that after further research, ghrelin may be used in some cases to improve motor learning. For this purpose, the authors suggest comparing the effects of these two drugs in old rats or with brain degenerative diseases so that ghrelin can be used as a prophylactic drug or treatment for motor impairment after combining the results. It closing, it should be noted that more medical and behavioral studies are warranted with a focus on humans to confirm our hypothesis.

Authors' contributions

HK, and VS contributed to conception and design. VS conducted to all experimental work, data and statistical analysis, and interpretation of data. HK was responsible for overall supervision. VS drafted the manuscript, which was revised by HK. All authors read and approved the final manuscript.

Ethical Approval

The Ethics Committee of Shahid Beheshti University confirmed the validity of this project (ethical code: IR.SBU.REC.1399.047).

Competing Interests

The authors declare that they have no conflict of interest.

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