

Uremia-caused changes of ghrelin system in hippocampus may be associated with impaired cognitive function of hippocampus

Zhian Lv¹ · Jie Gao¹ · Li Wang¹ · Zhao Chen¹ · Haozheng Yuan² · Xiaoqin Ma¹ · Jiamei Lu¹ · Jianrui Lv² · Xili Wu³ · Lei Zhang⁴ · Linting Wei¹ · Rongliang Xue² · Rongguo Fu¹ · Liqun Ma¹

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Abstract

Purpose Hippocampus plays an important role in spatial learning and memory. Ghrelin, a brain-gut peptide, participates in the mnemonic functions of hippocampus through its receptor growth hormone secretagogue receptor (GHS-R) distributed in hippocampus. This study was to investigate whether there was a correlation between the changes of ghrelin system in hippocampus and the spatial cognitive impairment caused by chronic renal failure (CRF).

Methods Sprague–Dawley rats (male, 180 ± 10 g, 7–8 weeks old) were randomly classified into CRF group and control group ($n = 18$ per group). The CRF model was constructed by 5/6 nephrectomy and the controls treated with sham operation. By the 8th week after the surgery, the spatial cognitive function of rats was assessed by Morris

water-maze test (MWM), the protein expression of ghrelin and GHS-R in the hippocampus by immunohistochemistry, and the mRNA expression by real-time PCR. Statistical analysis was performed using ANOVA, Student–Newman–Keuls- q test and Pearson correlation analysis, and $P < 0.05$ was considered significant.

Results Compared with the controls, the time spent in “platform” quadrant (TSPQ) of rats with CRF was decreased, but the escape latency (EL) was increased significantly in MWM, and meanwhile the protein and mRNA expression of ghrelin and GHS-R in hippocampus was also increased significantly ($P < 0.05$ or $P < 0.01$). Correlation analysis suggested that the TSPQ was negatively but the EL was positively correlated with the mRNA expression of ghrelin and GHS-R ($P < 0.01$).

Conclusion The CRF-caused changes of ghrelin system in hippocampus might be correlated with the CRF-caused cognitive function impairment.

Zhian Lv and Jie Gao contributed equally to this work.

✉ Rongguo Fu
pipifu@126.com

✉ Liqun Ma
maliquin-doctor@tom.com

¹ Department of Nephrology, Second Affiliated Hospital, Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, People's Republic of China

² Department of Anesthesia, Second Affiliated Hospital, Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, People's Republic of China

³ Department of Integrated Chinese Traditional and Western Medicine, Second Affiliated Hospital, Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, People's Republic of China

⁴ Medical Laboratory, Second Affiliated Hospital, Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, People's Republic of China

Keywords Chronic renal failure · Ghrelin · Hippocampus · Spatial cognitive function impairment

Introduction

Cognitive impairment is prevalent in the patients with chronic renal failure (CRF), especially in dialysis patients, presenting with impaired executive function and decreased learning ability and memory [1]. These deficits in cognition may impair the patients' capability to provide informed consent for medical procedures and hinder them to participate in necessary medical care, such as dietary modification and medication compliance [2]. It has been confirmed that hippocampus plays important roles in the learning, memory and spatial navigation [3] and uremia may trigger a certain

pathological changes in the hippocampus [4]. Ghrelin is a 28-amino acid brain-gut peptide mainly produced by the X/A-like endocrine cells of the gastric oxyntic mucosa and partly by the hypothalamic arcuate nucleus, pituitary, kidney and placenta [5]. By activating the functionally active, signal-transducing form of growth hormone secretagogue receptor (GHS-R), a G protein-coupled receptor, ghrelin has multiple biological functions, such as regulation of growth hormone secretion, energy balance, feeding and gastrointestinal motility, improvement of cell proliferation, and protection of neurons [6, 7]. Previous studies demonstrated that ghrelin may have a role in mnemonic functions of hippocampus through its receptor GHS-R distributed in the hippocampus [8, 9]. Our prior study demonstrated that the expression of ghrelin and its receptor GHS-R in hippocampus were obviously increased under the influence of uremia [10]. We hypothesized that the changes of ghrelin system in hippocampus might be associated with the cognitive impairment caused by CRF. Thereupon we conducted this study to testify the hypothesis.

Materials and methods

Experimental animals

Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University approved animal use for this current study. Animal care and treatment were conducted in conformity with the Guide for the Care and Use of Laboratory Animals. Sprague–Dawley (SD) rats (male, 180 ± 10 g, 7–8 weeks old) were purchased from Laboratory Animal Center in the Fourth Military Medical University and classified randomly into CRF group and control group ($n = 18$ per group). They were housed under standardized conditions in plastic cages (2 rats per cage, light–dark cycle 12/12 h, temperature 22 ± 2 °C, humidity 50 ± 10 %), fed with standard diet, and had free access to tap water. The rats experienced 3-day habituation to laboratory conditions, and all of them were normal. After that they were taken to the next experiments.

CRF model

5/6 nephrectomy (5/6 NX) was used to construct CRF model. The SD rats were anesthetized by intraperitoneal injection of 10 % chloral hydrate (3 mL/kg) and then placed in the right lateral position. Routine skin preparation and sterilization were followed by the opening of the left kidney of rats using an oblique dorsal incision at 1 cm inferior to rib edges and 1.5 cm to the left side of spine. Then the two poles of the left kidney were ablated, but adrenal glands were protected. Four days later, a unilateral

nephrectomy at the right side was done. The control rats were treated with sham operation that was conducted in the same way as 5/6 NX except the removal of renal tissue. After surgery all the rats were fed with normal diet in single cage and administered with 3 days of penicillin intraperitoneal injection (200,000 U per day).

Assays for CRF development

0.5–1 mL blood was sampled from rat tail vein every 2 weeks for detection of plasma creatinine (PCr) and blood urea nitrogen (BUN) using automatic biochemical analyzer (7170A, Hitachi, Japan). By the 8th week after the surgery, the PCr values in the CRF group were detected twice higher than the controls', and then Morris water-maze test was allowed to start.

Morris Water-maze test (MWM)

The main components of the test were a circular pool (diameter 150 cm) filled with water (depth 40 cm) that was maintained at 25.0 ± 2.0 °C and a rescue landing platform (diameter 6 cm) that was placed in one of four quadrants. During training, the platform was exposed, 0.5 cm above the water surface. Later, after the animal was trained and ready for testing, the platform was just below the surface of the water, 0.5 cm under the water surface, and was not visible because the water was made opaque with milk. The testing lasted 6 days and contained two sub-experiments: (a) place navigation test and (b) navigational memory test [11].

The place navigation test was designed to determine spatial learning ability and conducted in the first 5 days. Briefly, it was that the rats were put into the water in the quadrant opposite to the rescue landing platform (start position) and the time (s) taken by the rats to climb onto the rescue landing platform was recorded as escape latency (EL). Firstly, the rats experienced three consecutive training days during which each rat had four chances in succession to find the landing platform (0.5 cm above the water surface) within 120 s and had a rest (20 min) between swimming trials. If the rats could not find the platform within 120 s, the rats were gently guided to the platform to rest and the EL was recorded as the maximum value (120 s). On the 4th and 5th day, the rescue landing platform was placed 0.5 cm below the surface of the water opacified by milk and the EL recorded in the 2 days was taken as the data to stand for the spatial learning ability.

The navigational memory test was designed to evaluate the ability to remember the original position of the platform. On the 6th day, the rescue landing platform was removed from the pool. The rats were put into the start position and the time (s) that rats stayed in the

quadrant where the platform used to be within 120 s was recorded as time spent in “platform” quadrant (TSPQ). The mean swim speed within the 120 s was measured. Each rat had only one attempt. The animal behavior analysis system (ZS-001), including tracking software and video camera, was provided by Beijing Zhongshi Dichuang Science and Technology Development Co., Ltd. (Beijing, China).

Immunohistochemical analysis

Immunohistochemical (IHC) analysis of ghrelin expression in the hippocampus was started immediately after the navigational memory test. Rats ($n = 6$ per group) were fixed on an operating table in a supine position after anesthesia by intraperitoneal injection of 10 % chloral hydrate (3 mL/kg). The thoracic cavities of rats were opened to expose the pericardium, right ventricle and right atrial appendage. 80 mL of physiological saline and 400 mL of 4 % paraformaldehyde buffer were rapidly injected into the left ventricle and ascending aorta. Then, the rat's brain was removed and processed with the following steps: tissue fixation (8 h in 4 % paraformaldehyde buffer), rinsing tissue with purified water, dehydration through graded ethanol solutions, rapid freezing in liquid nitrogen, embedding tissues in paraffin blocks, cutting serial sections (thickness 10 μ m) and pasting the sections on polylysine-coated slides.

SABC immunohistochemical staining was employed. The primary antibodies of ghrelin were rabbit anti-ghrelin (Rat) antibody (1:2500). The secondary antibody was biotinylated goat anti-rabbit IgG (1:1000–3000). All antibodies were purchased from Phoenix Biotech Co., Ltd.

After DAB coloration and neutral gum mounting, we observed the protein expression of ghrelin in hippocampus (magnification 400 \times), randomly selected ten different high-power fields (HPF) within the hippocampus on the serial sections, and measured the gray value of each HPF using ImageJ. The mean of the 10 HPFs was used as expression index.

mRNA detection

Immediately after the navigational memory test, the rats ($n = 12$ per group) were anesthetized by intraperitoneal injection of 10 % chloral hydrate (3 mL/kg) and beheaded for hippocampus tissue collection. Their brains were quickly removed into a petri dish that was placed on an ice tray and contained PBS solution at 0–4 $^{\circ}$ C. The pia mater and arachnoid were carefully peeled off. The coronal brain sections (1–4 mm posterior to the chiasma opticum) were cut out. The hippocampal region was identified according to the Structure of the Rat Brain (3rd edn) by Larry

Swanson [12], and then 0.1 g brain tissue in the hippocampal region was taken from the sections randomly.

RNA isolation was performed using Trizol Kit (Tiangen Biotech (Beijing) Co., Ltd.) according to the manufacturer's instructions. After reverse transcription, the resulting materials were used for PCR amplification using gene-specific primer pairs and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Ghrelin and GHS-R mRNA were determined quantitatively by using Bio-Rad IcyLer iQTM (Bio-Rad, Hercules, CA, USA) and β -actin as intracontrast gene or internal control.

The sequences of β -actin primer were: upper primer: 5'-TCC TAG CAC CAT GAA GAT C-3' and lower primer: 5'-AAA CGC AGC TCA GTA ACA G-3'. The sequences of ghrelin primer were: upper primer: 5'-GAA AGG AAT CCA AGA AGC CA-3' and lower primer: Reverse 3'-GGA GCA TTG AAC CTG ATT TC-5'. The sequences of GHS-R primer were: upper primer: 5'-CGA CCT GCT CTA GCA AAC TC-3' and lower primer: 3'-CAC GCC CAC CAG CAC GAA GA-5'.

For real-time PCR, the amplification conditions of ghrelin were initial denaturation (95 $^{\circ}$ C, 3 min), 40 cycles of denaturation (95 $^{\circ}$ C, 10 s), annealing (60.5 $^{\circ}$ C, 10 s), extension (72 $^{\circ}$ C, 10 s), and then a final extension (72 $^{\circ}$ C, 10 min). The amplification conditions of GHS-R were initial denaturation (95 $^{\circ}$ C, 3 min), 40 cycles of denaturation (95 $^{\circ}$ C, 10 s), annealing (63 $^{\circ}$ C, 10 s), extension (72 $^{\circ}$ C, 10 s), and then a final extension (72 $^{\circ}$ C, 10 min).

Statistical analysis

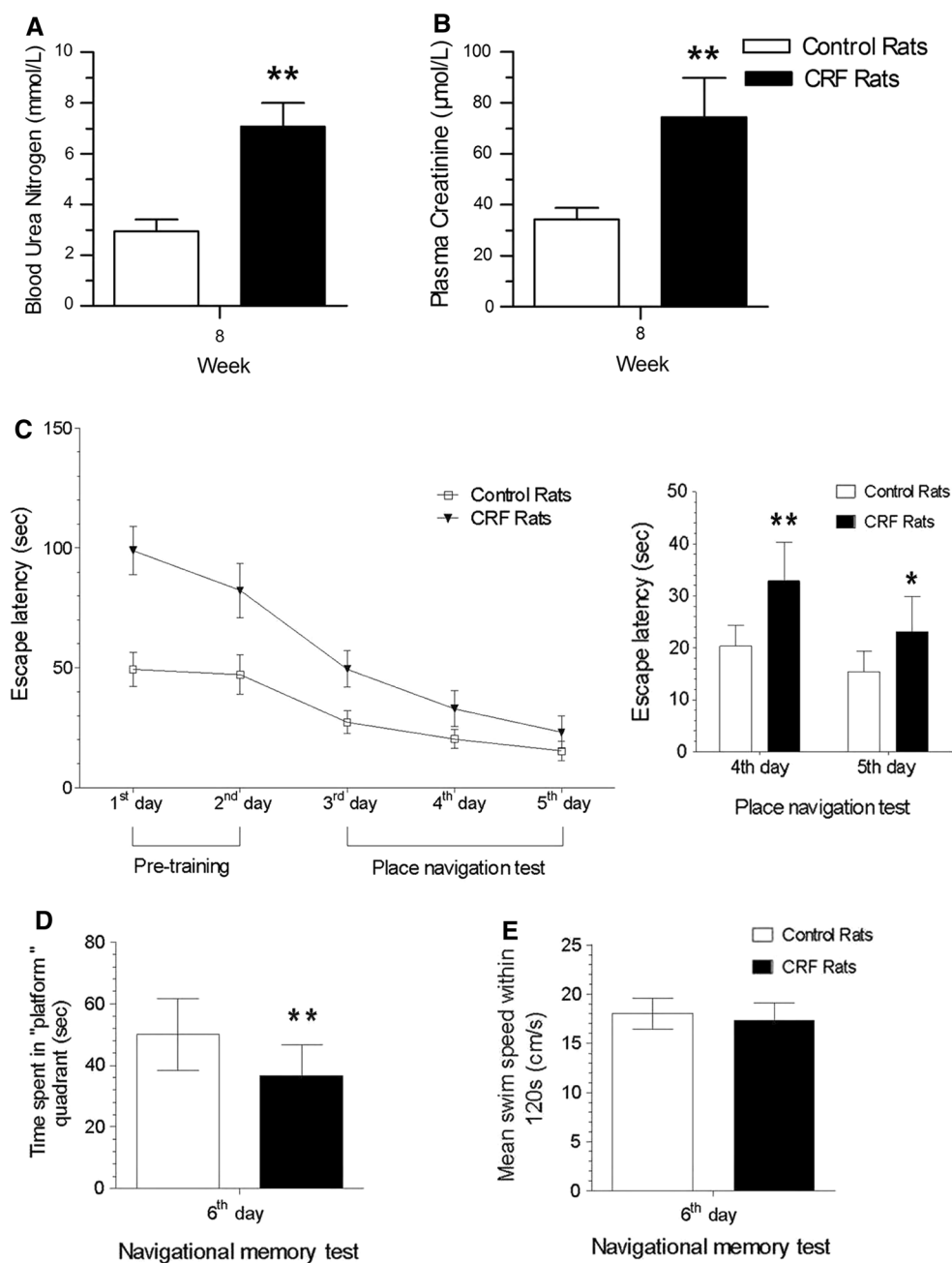
All data were expressed as mean \pm SD and processed with the statistics software package SPSS 13.0. After the Levene's test for homogeneity of variance of each group, the group differences were analyzed with ANOVA and the pairwise comparison with Student–Newman–Keuls q test (SNK- q). The correlation between the ghrelin and GHS-R mRNA expression and the EL and TSPQ was, respectively, analyzed by two-tailed Pearson correlation analysis. $P < 0.05$ would normally be considered significant.

Results

Before surgery, all the rats were healthy and no difference existed in BUN and PCr between two groups ($P > 0.05$). By the 8th week after the 5/6 NX, the BUN and PCr of CRF rats exceeded double the normal level ($P < 0.01$) (Fig. 1a, b).

In the pre-training for Morris water-maze test, all the rats of both groups could get on the rescue landing platform within 120 s without guidance, and the EL recorded were gradually decreased day by day, indicating that all

Fig. 1 Results of CRF model assays and MWM test. The BUN value (a) and the PCr value (b) of CRF rats are double more than the normal value by the 8th week after surgery. c The escape latency (EL) of both groups was recorded continuously in the first 5 days. The EL recorded in the first 3 days was the results of pre-training, and the EL on the 4th and 5th day was the formal results standing for the space navigation ability. d The time spent in “platform” quadrant (TSPQ) was recorded on the 6th day of the Morris water-maze test. e No significant differences are found in the mean swim speed within 120 s (navigational memory test)

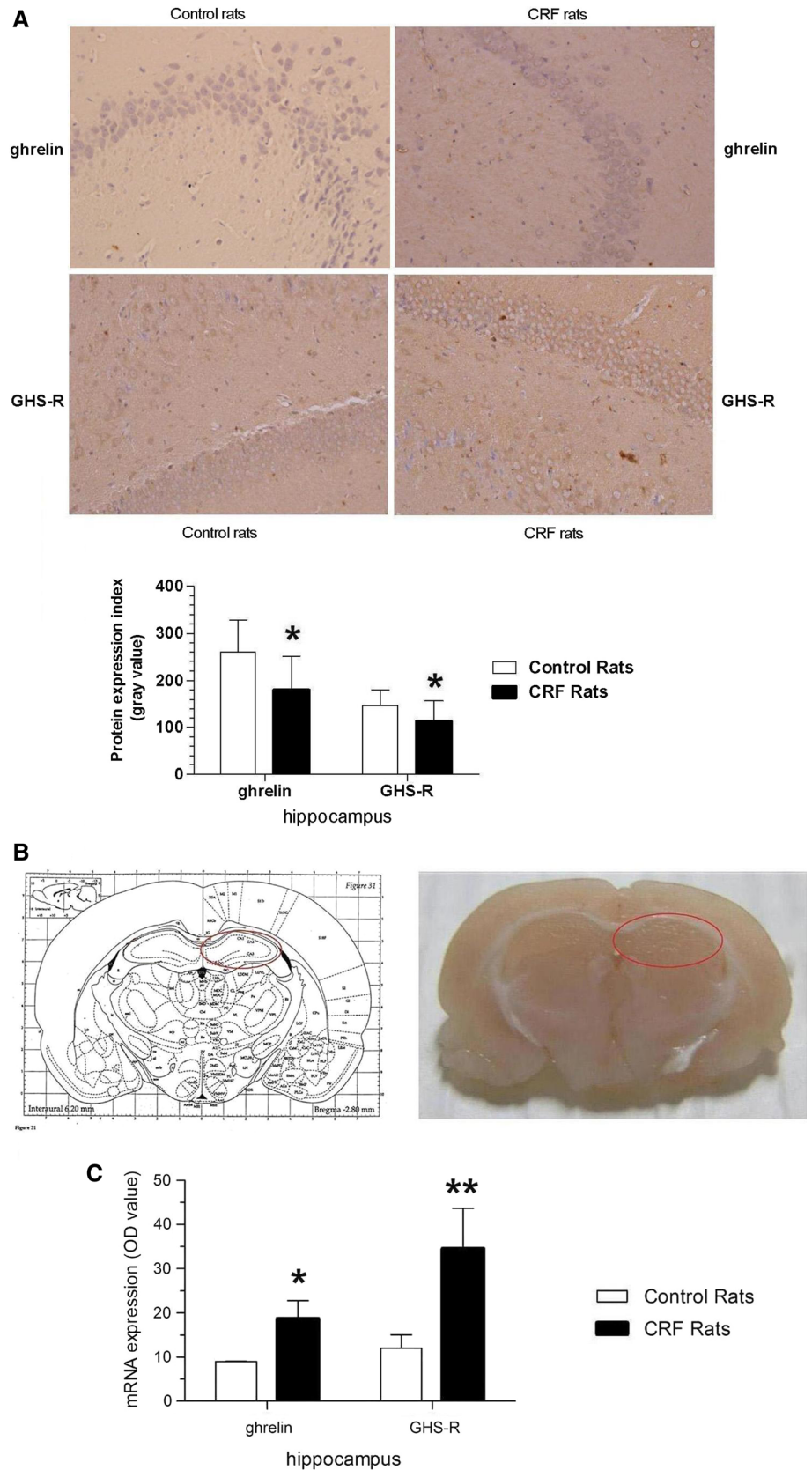


the rats had the spatial learning ability and had no problem in the visual acuity (Fig. 1c). The results of Morris water-maze test demonstrated the spatial learning and memory ability was decreased in the CRF group relative to the controls, presenting with the significantly increased EL and decreased TSPQ in the rats with CRF ($P < 0.05$ or $P < 0.01$) (Fig. 1c, d). The two groups had no significant difference in the mean swim speed measured in the navigational memory test ($P > 0.05$) (Fig. 1e).

On the IHC-stained sections, the ghrelin proteins presented with the yellow–brown granules distributed in the membrane of cells and/or cytoplasm of ghrelin positive

cells. And the GHS-R proteins, stained yellow–brown also, were located in the membrane of GHS-R-positive cells. It was found that the IHC-stained yellow–brown ghrelin granules were hardly detected in the hippocampal region of control rats, but they were detected in the hippocampus region of CRF rats. The comparison of ghrelin protein expression index between two groups showed that CRF group was significantly higher ($P < 0.05$). The IHC-stained GHS-R granules can be detected in the hippocampal region of both control and CRF rats. But statistics analysis showed that CRF rats were significantly increased relative to the controls ($P < 0.05$) (Fig. 2a).

Fig. 2 Results of protein and mRNA detection. **a** Ghrelin and its receptor GHS-R protein expression in the hippocampus of two groups are showed with magnification 400-fold. And the gray mean \pm SD of ghrelin and GHS-R in the two groups demonstrates the between-group differences in protein expression index. **b** According to the rat brain structure by Larry Swanson, the hippocampus is identified on the coronal section of rat brain. The *circled* region with *red line* is the hippocampus region for tissue sample collection. **c** The OD value (mean \pm SD) of ghrelin and GHS-R mRNA expression in the hippocampus of both groups. ****** $P < 0.01$; ***** $P < 0.05$



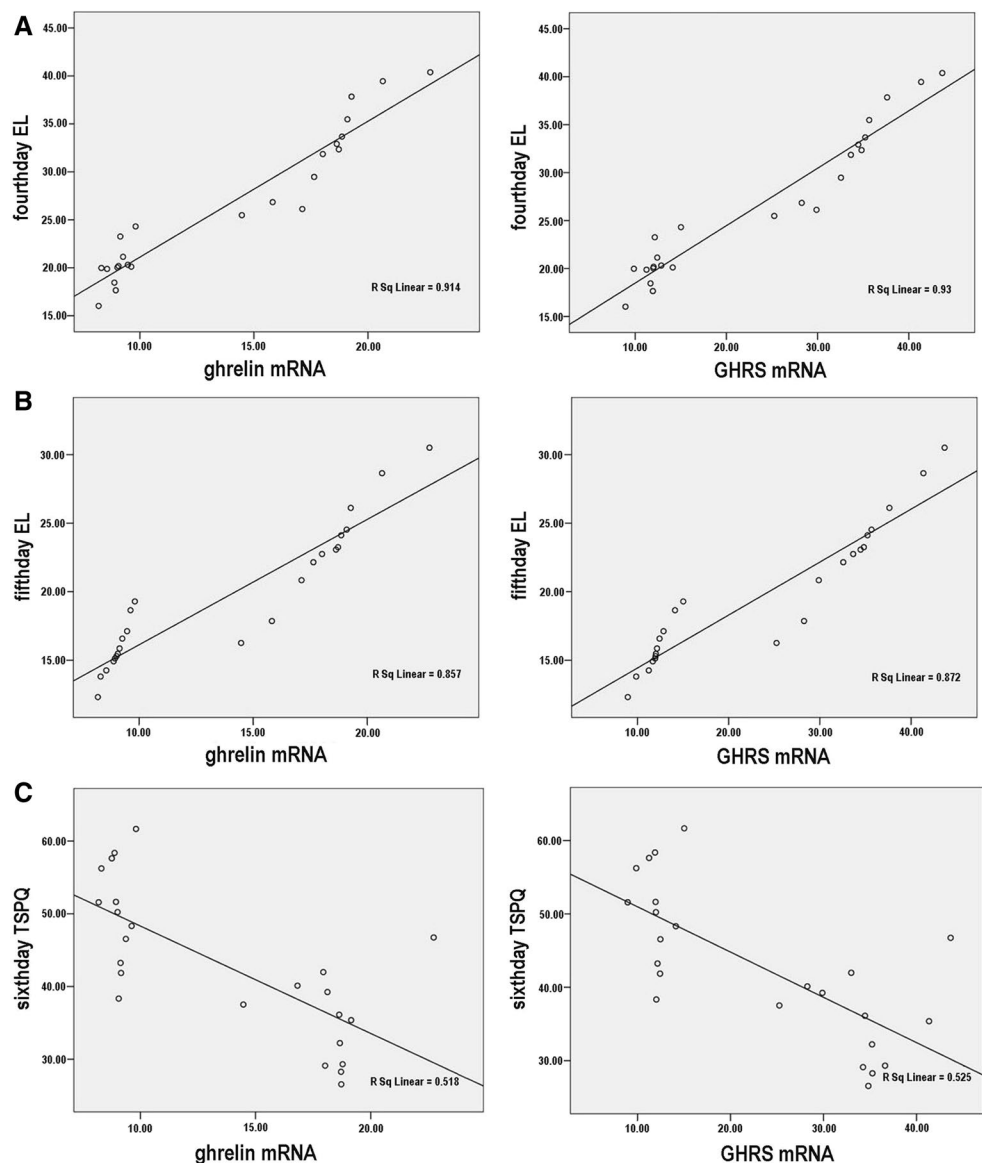
Bio-Rad IcyLer iQTM was used to read the optical density (OD) at 260 & 280 nm, and each sample was tested in triplicate. The ratio of OD260/OD280 nm of each sample ranged 1.8–2.0. Comparing with the controls, the OD value of ghrelin mRNA expression was increased in the hippocampus, likewise for the GHS-R mRNA expression ($P < 0.01$) (Fig. 2b, c). Apparently, the ghrelin and GHS-R mRNA expression was significantly up-regulated in the hippocampus of CRF group.

It was revealed in the correlation analysis that the hippocampal ghrelin and GHS-R mRNA expression was positively correlated with the EL recorded on the 4th day (Fig. 3a) and the EL on the 5th day (Fig. 3b) respectively, but negatively with the TSPQ recorded on the 6th day ($P < 0.01$) (Fig. 3c).

Discussion

The hippocampal function can change substantially at different points in the estrous cycle [13]. To avoid the adverse influence of menstrual cycle to hippocampus, this study only selected male rats. Although MWM is a classic behavioral test of spatial learning and memory in rats, the influence of motor dysfunction induced by uremia on MWM needs to be precluded [14]. This influence was precluded in this current study by the measurements of mean swim speed that were not different significantly between CRF rats and controls. Thus, the results of MWM can demonstrate that the rats with CRF had spatial cognitive impairment. And this finding is congruent with a previous study. Fujisaki's study confirmed that the mice with CRF had

Fig. 3 Correlation analysis. **a** The EL recorded on the 4th day is positively correlated with the ghrelin mRNA expression in hippocampus ($r = 0.956$; $P = 0.000 < 0.01$) and the GHS-R mRNA expression ($r = 0.964$; $P = 0.000 < 0.01$). **b** The EL recorded on the 5th day is positively correlated with the ghrelin mRNA expression ($r = 0.926$; $P = 0.000 < 0.01$) and with the GHS-R mRNA expression ($r = 0.934$; $P = 0.000 < 0.01$). **c** The TSPQ recorded on the 6th day is negatively correlated with the ghrelin mRNA expression ($r = -0.720$; $P = 0.000 < 0.01$) and with the GHS-R mRNA expression ($r = -0.725$; $P = 0.000 < 0.01$)



spatial working memory dysfunction by radial arm water maze and had no motor dysfunction by open field activity test in which the total number of line crossings was not different from controls [4]. Besides, clinical observation also demonstrates some uremic patients are concomitant with orientation and memory loss [2].

In central nervous system (CNS), the main site of ghrelin synthesis (albeit at much lower levels than the stomach) is the hypothalamus [15]. By immunocytochemical techniques, ghrelin expression was demonstrated in the inter-nuclear space between the lateral hypothalamus, the arcuate nucleus (ARH), the ventromedial nucleus (VMN), the dorsomedial nucleus (DMN), the paraventricular nucleus (PVN) and the ependymal layer of the third ventricle. In these areas, ghrelin was localized in axon terminals innervating the ARH, VMN, PVN, DMN and the lateral hypothalamus. These axons made synapses with neurons expressing NPY/AgRP and pro-opiomelanocortin. In hippocampus, the ghrelin expression is hardly detected [16], but its receptors GHS-R can be detected in the area [8]. Consistent with it, this study found that the ghrelin protein expression was also hardly detected in the hippocampus of control rats. Yet in the CRF rats, the ghrelin expression was obvious and clear, and its receptor expression was significantly higher, indicating that uremia did change the ghrelin system in the hippocampus.

Our correlation analysis suggests that the spatial cognitive impairment and the changes of ghrelin system in hippocampus caused by uremia may be correlated with each other. This is in line with previous studies on hippocampus and on ghrelin in hippocampus.

Previous studies have proven that hippocampus is a key region processing spatial navigation and memory consolidation [17]. Hippocampus has three main subregions: dentate gyrus (DG), CA3, and CA1. The CA1 has a unique anatomical position receiving the information simultaneously both from the DG–CA3 network and directly from the entorhinal cortex [18], and it may function as a mismatch processor in detecting spatial novelty [19]. The CA3 may play a role in encoding and retrieval of associations and in detecting the mismatch between the memory for the spatial context of each item and the current sensory input about the spatial position of the item. Under the mediation of entorhinal cortex to the overall learning of associations, the CA3 and DG could specifically encode the relationship between objects dependent on the relative times or locations at which they were encountered, acting an important role in the process of detecting the mismatch when a familiar object is placed in a new spatial location [20].

Ghrelin may have a role in the memory function of hippocampus [9, 21]. Carlini and colleagues proved that intracerebroventricular injections of ghrelin increased memory

retention. Subsequently they defined the site of peptide action more precisely by repeating intraparenchymal injections of increasing concentrations of the hormone in hippocampus, amygdala and dorsal raphe nucleus, and they observed a dose-dependent increase of memory retention in each condition with maximal effect in the hippocampus [22]. In addition, a study by Diano [23] confirms that ghrelin may control the hippocampal spine synapse density and memory performance. It was suggested that the effects of ghrelin on memory could depend on the availability of serotonin, since a serotonin uptake inhibitor (fluoxetine) might decrease both short- and long-term memory retention [24]. Yet we think the effects are more closely dependent on ghrelin receptors distributed in the hippocampus [8]. It is not only because there is the D1/GHS type 1a co-expression in hippocampus but also because the ghrelin receptors positively interact with dopamine and serotonin receptors in hippocampus [25].

It seems to be contradictory between the up-regulation of hippocampal ghrelin system and the spatial cognitive impairment induced by uremia because almost all publications cited above support that ghrelin improves rather than impairs hippocampus-related learning, including spatial learning. In other words, CRF-induced elevated expression of ghrelin and its receptor as found here should result in better water maze learning. However, we do not think it is contradictory because what we compared was the difference in spatial learning between CRF rats and health control rats, rather than the difference between CRF rats with different expressions of ghrelin system. We surmise that among the CRF rats, those with higher expression of ghrelin and its receptor in hippocampus would have better results in MWM. Of course, it needs more experiments to testify. Meanwhile we think that the seemingly contradiction may disclose a more complicated pathophysiological process induced by uremia.

As we know, the causes of cognitive impairment induced by CRF are complicated, involving various factors such as uremic encephalopathy, complications of dialysis procedure, high prevalence of clinical and subclinical cerebrovascular disease and various comorbidities (anemia, hypertension, diabetes, malnutrition, etc.) [26]. Similarly, multiple factors are involved in the up-regulation of ghrelin and its receptor in hippocampus, such as uremic toxins, inflammatory cytokines and central oxidative stress [27]. It has been confirmed that the main source of ghrelin acting on central neurons is from the peptide synthesized in the stomach and released into the general circulation [22], and the reduced renal excretion of ghrelin is the main causative factor to the increase of plasma ghrelin [28]. Besides, the model constructed in Diano's study implies that circulating ghrelin can reach significant concentrations in hippocampus [23]. It means circulating ghrelin may be contributive

to the up-regulation of ghrelin protein expression in the hippocampus of rats with CRF. But it is hard to use the circulating ghrelin to explain the up-regulation of mRNA expression of ghrelin and its receptor in hippocampus. Moreover, our prior studies proved that the same increased circulating ghrelin did not make the ghrelin and its receptor expression up-regulated but down-regulated in the hypothalamus and amygdala in the same CRF model rats [10, 29]. Up to now, it is still an enigma.

We speculate that this process might be a process of adaptive change or a process of functional compensation, namely the gradually up-regulated ghrelin system in hippocampus compensates the cognitive function of hippocampus impaired by uremia. We reasoned that it has been confirmed that peripheral ghrelin injections may rapidly rearrange synaptic organization with an increase in spine density in CA1 regions of hippocampus, and the promotion of ghrelin to long-term potentiation has a positive correlation with spatial memory and learning [23]. Besides, it is suggested that since the loss of cognitive functions in aging has been supposed to involve a decline in dopamine or serotonin signaling [30, 31], ghrelin potentiation of the neurotransmitters in hippocampus may represent an interesting mechanism to intervene on memory impairment due to senescence or Alzheimer disease [22, 23].

The limitation of this study was to overlook the influence from blood pressure and hemoglobin concentration on cognitive performance, because the two factors can be affected by uremia. In spite of that, we think that it may be concluded after considering all above that the changes of ghrelin system in hippocampus are correlated with the spatial cognitive impairment in the rats with CRF and the changes might be a functional compensation mechanism for the spatial cognitive impairment caused by CRF.

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Compliance with ethical standards

Conflict of interest No conflict of interest was declared.

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