Ghrelin is required for dietary restriction-induced enhancement of hippocampal neurogenesis: lessons from ghrelin knockout mice

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Abstract. Neurogenesis occurs in the adult hippocampus and is enhanced by dietary restriction (DR), and neurogenesis enhancement is paralleled by circulating ghrelin level enhancement. We have previously reported that ghrelin modulates adult neurogenesis in the hippocampus. In order to investigate the possible role of ghrelin in DR-induced hippocampal neurogenesis in adult mice, ghrelin knockout (GKO) mice and wild-type (WT) mice were maintained for 3 months on DR or ad libitum (AL) diets. Protein levels of ghrelin in the stomach and the hippocampus were increased by DR in WT mice. One day after BrdU administration, the number of BrdU-labeled cells in the hippocampal dentate gyrus was decreased in GKO mice maintained on the AL diet. DR failed to alter the proliferation of progenitor cells in both WT and GKO mice. Four weeks after BrdU injection, the number of surviving cells in the dentate gyrus was decreased in AL-fed GKO mice. DR increased survival of newborn cells in WT mice, but not in GKO mice. Levels of brain-derived neurotrophic factor protein in the hippocampus were similar between WT and GKO mice, and were increased by DR both in WT and GKO mice. These results suggest that elevated levels of ghrelin during DR may have an important role in the enhancement of neurogenesis induced by DR.

Key words: Ghrelin, Adult neurogenesis, Hippocampus, Dietary restriction

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Abstract. Neurogenesis occurs in the adult hippocampus and is enhanced by dietary restriction (DR), and neurogenesis enhancement is paralleled by circulating ghrelin level enhancement. We have previously reported that ghrelin modulates adult neurogenesis in the hippocampus. In order to investigate the possible role of ghrelin in DR-induced hippocampal neurogenesis in adult mice, ghrelin knockout (GKO) mice and wild-type (WT) mice were maintained for 3 months on DR or ad libitum (AL) diets. Protein levels of ghrelin in the stomach and the hippocampus were increased by DR in WT mice. One day after BrdU administration, the number of BrdU-labeled cells in the hippocampal dentate gyrus was decreased in GKO mice maintained on the AL diet. DR failed to alter the proliferation of progenitor cells in both WT and GKO mice. Four weeks after BrdU injection, the number of surviving cells in the dentate gyrus was decreased in AL-fed GKO mice. DR increased survival of newborn cells in WT mice, but not in GKO mice. Levels of brain-derived neurotrophic factor protein in the hippocampus were similar between WT and GKO mice, and were increased by DR both in WT and GKO mice. These results suggest that elevated levels of ghrelin during DR may have an important role in the enhancement of neurogenesis induced by DR.

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Ghrelin is an endogenous ligand of GH secretagogue receptor (GHS-R) 1a. It is a unique GH-releasing 28-aminoacid n-octanoiated peptide that is mainly synthesized and released from the stomach [1]. In addition to its major effects on GH release and energy homeostasis [2, 3], ghrelin also has diverse peripheral effects [4]. Furthermore, several lines of evidence indicated that ghrelin acts in the central nervous system to control neuronal functions and subsequently alter many brain functions [5]. We have reported that ghrelin stimulates the proliferation of newborn cells in the hippocampus of adult mice and systemic administration of anti-ghrelin antiserum reduces the proliferation of hippocampal progenitor cells in the subgranular zone (SGZ) [6]. Additionally, in our recent report [7], we found that mice with targeted disruption of the ghrelin gene showed a decreased number of progenitor cells in the dentate gyrus (DG) of the hippocampus, while ghrelin replacement restored progenitor cell numbers to those of wild-type (WT) controls. Proliferative effect of ghrelin on hippocampal progenitor cells seems to be mediated through the activation of its receptor because GHS-R1a antagonist completely blocked the effect of ghrelin in vitro [8]. Collectively, these findings suggest that ghrelin may play an important role in adult hippocampal neurogenesis.

It is now well documented that dietary restriction (DR) gives beneficial effects like increasing life span, delaying the onset of age-associated diseases, and reducing oxidative stress and damage [9]. Previous data suggest that DR increases adult hippocampal neurogenesis by increasing the survival of neural stem cells [10-12] and enhances synaptic plasticity [13], which play important roles in learning and memory [14].
The precise mechanisms responsible for the enhancing effects of DR on hippocampal neurogenesis remain elusive. Cultured HeLa cells incubated in the presence of serum from DR rats caused an increase in mitochondrial bioenergetic capacity, mitochondrial biogenesis and reduced reactive oxygen species production when compared with cells incubated with serum from ad libitum (AL)-fed rats [15]. These findings indicate that certain factors or hormones, which are increased during DR, may be responsible for the beneficial effects of DR. Considering that plasma ghrelin levels rise significantly during DR [16, 17], this peptide hormone may be a potential mediator of enhancement of hippocampal neurogenesis induced by DR. However, it is still unclear whether ghrelin is involved in DR-induced hippocampal neurogenesis. Therefore, we employed mice lacking ghrelin to directly examine if the augmentation of hippocampal neurogenesis in response to DR is mediated by ghrelin.

Materials and Methods

Animals and BrdU injection

To determine the role of ghrelin on DR-induced hippocampal neurogenesis in young adult mice, we used 8-10 week-old male GKO mice [18] and age-matched WT C57BL/6J mice. The animals were housed under controlled 12:12 h light-dark cycle environmental conditions. They were habituated to the housing conditions for 7-10 days prior to the beginning of the experimental procedures. WT and GKO mice were divided into two groups, an AL group which had been free access to food, and a DR group which was maintained on every-other-day feeding regimen (24-h fast followed by 24-h fed period) for 3 months. All experiments were approved by the Kyung Hee University Animal Care Committee and conducted according to the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of health. All efforts were made to minimize animal suffering and to reduce the number of animals used. BrdU (50 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) was given intraperitoneally once a day for 12 days [10] and mice were transcardially perfused 1 or 28 day(s) after the last BrdU administration (for the evaluation of BrdU-labeled cells). Subsets of mice in each diet group were processed for the analysis of protein levels of ghrelin as described below.

Tissue preparation and BrdU immunohistochemistry

Tissue preparation and BrdU immunohistochemistry were performed as we previously described [7]. Briefly, animals were anesthetized with xylazine and ketamine and then perfused transcardially with a freshly prepared solution of 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were removed and post-fixed overnight in the same fixative before being immersed in a solution of 30% sucrose in PBS. Serial 30-μm-thick coronal tissue sections were cut using a microtome and stored in cryoprotectant (25% ethylene glycol, 25% glycerol, 0.05 M PB; pH 7.4) at –20 °C for later immunohistochemistry procedures. In order to minimize staining variations, the brain sections from the same experiment were processed for immunohistochemical staining simultaneously. Free-floating tissue sections were then incubated overnight at 4 °C with primary rat anti-BrdU antibody (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The sections were incubated with biotinylated secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA) and then visualized using the avidin–biotin–peroxidase complex method with diaminobenzidine tetrahydrochloride (DAB) as the chromogen. To ensure the detection of BrdU-labeled nuclei, we denatured the DNA before incubation with BrdU antibody because BrdU is incorporated into the DNA. DNA denaturation was performed in the following manner: tissue was incubated in 50% formamide and 2× SSC (1× SSC, 0.3 M NaCl and 0.03 M sodium citrate) for 2 h at 65 °C, rinsed for 15 min in 2× SSC, incubated again for 10 min in 0.1 M boric acid at pH 8.5. For immunodetection of BrdU, the DAB-nickel enhancement technique was used.

Image processing

Sections were examined by using a computer-assisted image analysis system consisting of a Zeiss Axioscope-2 microscope equipped with a computer-controlled motorized stage, a video camera, and Stereo Investigator software (MicroBrightField, Williston, VT, USA). For confocal analysis, sections were examined with a Carl Zeiss LSM 700 Meta (Oberkochen, Germany) confocal microscope equipped with appropriate objectives and excitation and emission filters. Digital images from the microscopy were slightly modified to optimize for image resolution, brightness and contrast using Adobe Photoshop CS5 software (Adobe Systems, San Jose, CA, USA).
Results

**DR increases gastric and hippocampal ghrelin protein levels in WT mice**

Young adult WT and GKO male mice were maintained on either an AL or a DR feeding regimen, where they were fed every other day: their body weights after 3 months on the diets were: WT AL, 28.2 ± 0.5 g; WT DR, 24.4 ± 0.4 (P < 0.05 vs. WT AL); GKO AL, 28.5 ± 0.5; GKO DR, 24.0 ± 0.4 (P < 0.05 vs. GKO AL) (Fig. 1A). As expected, the DR regimen decreased the body weights of WT mice as well as GKO mice. In WT mice, ghrelin protein levels in the stomach were significantly increased by DR compared to AL-fed mice (Fig. 1B). Ghrelin was barely detectable in the hippocampus of WT mice, while the DR regimen increased ghrelin protein levels (Fig. 1C). Ghrelin expression was not detected in GKO mice, as expected.

**DR enhances hippocampal neurogenesis in WT mice but not in GKO mice**

At 1 day after the last BrdU administration, the numbers of BrdU-labeled cells in the DG of WT mice maintained on AL and DR diet regimens were not significantly different, as shown in Fig. 2. However, the number of BrdU-labeled cells at this time point in GKO mice maintained on DR was not different than in GKO AL mice. In WT mice, ghrelin protein levels in the stomach were significantly increased by DR compared to AL-fed mice (Fig. 1B). Ghrelin was barely detectable in the hippocampus of WT mice, while the DR regimen increased ghrelin protein levels (Fig. 1C). Ghrelin expression was not detected in GKO mice, as expected.

**Quantification**

The total numbers of BrdU-labeled cells in mice were manually counted in the right and left SGZ along the superior and inferior blades of the DG of every sixth sections throughout the entire extent of the hippocampus (from Bregma -1.06 mm to Bregma -3.80 mm), using Stereo Investigator (MicroBrightField, Williston, VT) as previously described [7]. Analysis was performed using a computer-assisted image analysis system consisting of a Zeiss Axioscope-2 microscope equipped with a computer-controlled motorized stage, a video camera, and Stereo Investigator software. Counting was done at 400× magnification using a Zeiss Axioscope-2 microscope equipped with a 40× objective lens. Cells were counted throughout each section by focusing through each focal plane of the section to ensure that all BrdU-labeled cells were visible. The resulting numbers from each section were added and multiplied by 6 to obtain the estimated total number of labeled cells per DG for each brain.

**Western blot analysis**

Tissues were lysed in a buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 140 mM NaCl, 1% (w/v) Nonidet P-40, 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 10 µg/ml aprotinin. Tissue lysates were separated by 12% SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membranes were soaked in blocking buffer (1× Tris-buffered saline, 1% BSA, 1% nonfat dry milk) for 1 h and incubated overnight at 4 °C with rabbit anti-ghrelin (Chemicon International, Inc., Temecula, CA, USA), rabbit anti-brain-derived neurotrophic factor (BDNF) (Santa Cruz Biotechnology) or rabbit anti-β-actin (Abcam Inc, Cambridge, UK) antibody. Blots were developed using a peroxidase-conjugated anti-rabbit IgG and a chemiluminescent detection system (Santa Cruz Biotechnology). The bands were visualized using a ChemiDoc XRS system (Bio-Rad) and quantified using Quantity One imaging software (Bio-Rad).

**Statistical analysis**

Data are presented as mean ± SEM. Comparisons between AL and DR groups were made by unpaired Student’s t test. Statistical analysis between multiple groups was performed using 1-way ANOVA and Holm-Sidak method for multiple comparisons using SigmaStat for Windows Version 3.10 (Systat Software, Inc. Point Richmond, CA, USA). P < 0.05 was considered statistically significant.
Fig. 1  Protein levels of ghrelin in the stomach and the hippocampus are increased in WT mice maintained on a DR regimen. A, Body weights of mice after 3 months on either an AL or a DR diets in WT and GKO mice. B and C, Effect of DR on ghrelin protein levels in the stomach (B) and the hippocampus (C) assessed by Western blot. Ghrelin band intensity was normalized to β-actin band intensity. The data expressed as the mean ± SEM. *, \(P < 0.05\) vs. AL.

Fig. 2  DR does not increase cell proliferation in the DG both in WT and GKO mice. A, Photomicrographs of BrdU-labeled cells in the hippocampus 1 day after BrdU administration in WT (a and b) and GKO (c and d) mice that had been maintained on either AL (a and c) or DR (b and d) for 3 months. Scale bar represents 100 μm. B, Quantitative analysis of BrdU-labeled cells in the hippocampus. The data expressed as the mean ± SEM. Values with no letters (a, b) in common are significantly different from each other (\(P < 0.05\)).
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Fig. 3 DR increases survival of newly generated cells in the DG in WT mice but not in GKO mice. A, Photomicrographs of BrdU-labeled cells in the hippocampus 28 day after BrdU administration in WT (a and b) and GKO (c and d) mice that had been maintained on either AL (a and c) or DR (b and d) for 3 months. Scale bar represents 100 μm. B, Quantitative analysis of BrdU-labeled cells in the hippocampus. The data expressed as the mean ± SEM. Values with no letters (a, b, c) in common are significantly different from each other ($P < 0.05$).

Fig. 4 DR increases hippocampal BDNF protein levels in both WT and GKO mice. BDNF protein levels were determined by Western blot analysis. BDNF band intensity was normalized to β-actin band intensity. The data expressed as the mean ± SEM. Values with no letters (a, b) in common are significantly different from each other ($P < 0.05$).

Discussion

In the current study, we provide evidence that ghrelin is involved in DR-induced hippocampal neurogenesis in adult mice. Specifically, DR increased the protein levels of gastric and hippocampal ghrelin in WT mice. Our data also show that DR promotes the hip-
It has been reported that hippocampal BDNF levels were increased by DR in WT mice and to a lesser amount in BDNF +/- mice [11], suggesting the role of BDNF in the enhancement of hippocampal neurogenesis by DR. In the current study, no significant differences in basal expression levels of BDNF in the hippocampus were observed between WT and GKO mice. Three months of DR increased the levels of BDNF in WT mice, supporting a role of BDNF in DR-induced hippocampal neurogenesis. BDNF protein levels were also increased in GKO mice maintained on DR. These data suggest that the mechanism whereby DR induces hippocampal BDNF production is not affected by ghrelin. We assume that factors other than ghrelin are involved in DR-induced augmentation of hippocampal BDNF expression in GKO mice. Surprisingly, in GKO mice, DR failed to increase the number of BrdU-labeled cells in spite of enhanced expression of BDNF. Our explanation for this discrepancy might be that ghrelin may be required for BDNF-mediated increase in hippocampal neurogenesis under the DR conditions. It also has been suggested that another neurotrophic factor, neurotrophin-3 (NT-3), may be involved in DR-induced neurogenesis. DR upregulated NT-3 in the hippocampus and increased hippocampal plasticity and neurogenesis [10]. In addition, interferon-γ (IFN-γ), which is upregulated during DR [26], has been shown to promote neurogenesis in the hippocampus [27]. DR-induced changes in the expression of NT-3 and IFN-γ in the hippocampus of mice lacking ghrelin remain to be determined.

In conclusion, our findings suggest that the mechanism by which DR promotes adult hippocampal neurogenesis by increasing survival of newborn cells and this effect may be dependent on ghrelin signaling.

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**Disclosure Summary**

The authors have nothing to disclose.
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