RAPID COMMUNICATION

Ghrelin Regulates Hippocampal Neurogenesis in Adult Mice

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Abstract. The aim of our study was to investigate the effect of the peripheral administration of ghrelin, a peptide hormone secreted from the stomach, on cellular proliferation and differentiation of progenitor cells in the adult hippocampus. Double immunohistochemical staining revealed that Ki-67-positive hippocampal progenitor cells expressed ghrelin receptors. In mice treated with ghrelin (80 µg/kg, i.p.) for 8 days, bromodeoxyuridine incorporation and doublecortin-positive neuroblasts were significantly increased in the dentate subgranular zone. We also found that the numbers of bromodeoxyuridine- and doublecortin-immunoreactive cells were significantly reduced after anti-ghrelin antibody (10 µg/kg, i.p.) treatment for 8 days. Therefore, our results indicate that ghrelin induces proliferation and differentiation of adult hippocampal progenitors, suggesting an involvement of ghrelin in hippocampal neurogenesis.

Key words: ghrelin, neurogenesis, hippocampus, BrdU, doublecortin

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NEUROGENESIS is a process of generating functionally integrated neurons from progenitor cells. Now it is well known that active neurogenesis occurs through life in several species, including humans, in the subventricular zone of the lateral ventricle and in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus [1, 2]. Hippocampal neurogenesis is of particular interest because the role of this structure is important for learning and memory [3]. Various factors such as age, growth factors, excitatory input, adrenal steroids, seizures, and enrichment of the environment are reported to affect neurogenesis of adult hippocampal neural stem cells [1, 2]. However, the endogenous factors that regulate adult hippocampal neurogenesis need to be better identified.

Ghrelin was discovered in 1999 as the endogenous ligand for growth hormone (GH) secretagogue (GHS) receptor 1a (GHS-R1a) [4]. Ghrelin is a unique peptide hormone esterified with octanoic acid on Ser3 that

is principally released from Gr cells in the oxyntic mucosa of the stomach [5]. In addition to stimulating GH release via the hypothalamus and direct pituitary pathways and inducing a positive energy balance by stimulating food intake while decreasing fat use through GH-independent mechanisms, ghrelin has been suggested to have numerous peripheral actions including direct effects on exocrine and endocrine pancreatic functions, carbohydrate metabolism, the cardiovascular system, gastric secretion, stomach motility, and sleep [6]. It has been reported that maternal ghrelin plays an important role in rat fetal development during pregnancy [7]. Ghrelin is also involved in neurogenesis of the rat fetal spinal cord [8]. In addition, in the nucleus of the solitary tract (NTS) [9] and the dorsal motor nucleus of vagus (DMNV) [10] in adult rats with cervical vagotomy, ghrelin promotes neural proliferation *in vivo* and *in vitro*. Moreover, very recently it has been reported that ghrelin increases cellular proliferation of adult rat hippocampal progenitor cells *in vitro* [11]. However, there is no report to date about the effect of ghrelin on neurogenesis in the adult mammalian SGZ of the DG *in vivo*. In the present study, we wanted to study the impact of systemically administered ghrelin. Therefore, the aim of this study was to investigate the proliferation and differentiation of

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progenitor cells in the adult DG in mice treated with ghrelin. We also investigated the role of endogenous ghrelin in adult neurogenesis by neutralizing ghrelin effect using anti-ghrelin antibody.

Methods

Male C57Bl/6 mice (22–24g, eight-week-old) were used in the present study. They were housed under controlled environmental conditions (12-h light and 12-h dark) and acclimated for at least 1 week. Food and tap water were available *ad libitum*. Animals were randomly assigned to groups of 5 animals. Ghrelin (Phoenix Pharmaceuticals, Burlingame, CA), rabbit anti-ghrelin antibody (Phoenix Pharmaceuticals) and normal rabbit IgG (Phoenix Pharmaceuticals) were dissolved in 0.9% normal saline. Ghrelin (80 µg/kg) or saline and anti-ghrelin antibody (10 µg/kg) or normal rabbit IgG (10 µg/kg) were injected intraperitoneally (i.p.) into mice once daily for 8 days. Body weight and food intake were measured daily during the period of treatment. All experiments were approved by the Kyunghee University Animal Care Committee and conducted according to the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of mice used.

To determine the proliferation of the neural precursor cells, mice were treated with 5-bromo-2'-deoxyuridine (BrdU; 50 mg/kg, i.p.) daily during the first five days of ghrelin treatment. Mice 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.

For immunohistochemical analysis of BrdU and doublecortin (DCX), four brain sections of each mice was taken from the region between bregma –1.7 mm and bregma –2.3mm; this was performed for each brain [Paxinos atlas]. To ensure the detection of BrdU-labeled nuclei, we denatured the DNA before

incubation with BrdU antibody. DNA denaturation was performed in the following manner: tissue was incubated in 50% formamide and 2X SSC (1X SSC, 0.3 M NaCl and 0.03 M sodium citrate) for 2 hr at 65°C, rinsed for 15 min in 2X SSC, incubated again for 30 min in 2 M HCl at 37°C, and rinsed again for 10 min in 0.1M boric acid at pH 8.5. Tissue sections were then incubated overnight at 4°C with primary antibodies (rat anti-BrdU antibody, 1:1,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; goat anti-DCX antibody, 1:1,000, Santa Cruz Biotechnology, Inc.). The sections were incubated with appropriate biotinylated secondary antibody (1:200, Vector Laboratories, Burlingame, CA) and then visualized using the avidin–biotin–peroxidase complex method with diaminobenzidine tetrahydrochloride (DAB) as the chromogen. For immunodetection of BrdU, the DAB–nickel enhancement technique was used.

The numbers of BrdU and DCX-immunostained cells were counted in the inner rim, defined as the border between the hilus and granule layers, of the granule cell layer (GCL) of DG, as previously described [12]. The inner rim length of GCL was measured using Stereo Investigator software (MicroBrightField, Williston, VT). All numbers are based on the numbers of individual cell nuclei. Counting was done with a 40X objective on a Zeiss Axioscope-2 microscope.

To determine if GHS-R1a was expressed in proliferating progenitor cells in DG of hippocampus, double immunofluorescence staining was performed. Free-floating sections were incubated with 5- (and 6-) carboxyfluorescein labeled anti-GHS-R1a antibody (1:500, rabbit, Phoenix Pharmaceuticals) and anti-Ki-67 antibody (1:1,000, rat, Abcam, Cambridge, UK) overnight at 4°C. After washing, the sections were incubated with a secondary Cy3-goat anti-rat IgG (1:400, Jackson ImmunoResearch, West Grove, PA) at room temperature for 1 h 30 min. Sections were counterstained with DAPI before mounting and images were acquired by the Carl Zeiss LSM 510 Meta (Oberkochen, Germany) confocal microscope.

Data are expressed as mean ± SEM. Experiments were repeated at least twice. Statistical analysis between groups was performed using one-way analysis of variants (ANOVA) and the Holm-Sidak method for multiple comparisons using SigmaStat for Windows Version 3.10 (Systat Software, Inc., Point Richmond, CA). A *p*-value < 0.05 was considered statistically significant.

Results

GHS-R1a expression in adult hippocampal progenitor cells

To examine whether adult hippocampal progenitor cells express GHS-R1a, we immunostained brain sections with GHS-R1a and Ki-67 (a marker for quantitative analysis of cell proliferation of SGZ precursors) [13] antibodies. Fig. 1 shows that GHS-R1a immunoreactivity was detected in dentate granule cells and SGZ of the hippocampus. Moreover, we also found that Ki-67 immunoreactivity was colocalized with GHS-R1a immunoreactivity. These results indicate that GHS-R1a is expressed in Ki-67-positive hippocampal progenitor cells.

Systemic administration of ghrelin stimulates proliferation and neuronal differentiation of adult hippocampal progenitor cells in the SGZ

The number of newly generated cells in the SGZ was determined by monitoring the incorporation and subsequent immunohistochemical detection of BrdU, a marker for mitotic cells [14], within the nuclei of dividing cells (Fig. 2A). BrdU-immunostained nuclei were located along the innermost region of the GCL, adjacent to the hilus. The nuclei were generally condensed and exhibited variable shape. In animals treated with ghrelin for 8 days, the number of BrdU-immunoreactive cells in the SGZ (50.4 ± 1.5 cells/mm) was significantly higher than in vehicle-treated animals (33.5 ± 1.6 cells/mm; $p < 0.01$) (Fig. 2A and C). In the present study, we chose 80 μ g/kg of ghrelin on the basis of our previous report that this dose of ghrelin exerted a potent neuroprotective action in the rats exposed to transient focal cerebral ischemia [15].

To determine whether exogenous ghrelin also affects neuronal differentiation in the SGZ, we measured the number of DCX, a marker of early neuronal differentiation [16]. The number of DCX-positive cells in the SGZ of ghrelin-treated mice (50.4 ± 1.7 cells/mm) was significantly higher than in the saline-treated mice (41.5 ± 1.3 cells/mm; $p < 0.01$) (Fig. 2B and D). Taken together, these results indicate that exogenous ghrelin stimulates proliferation and neuronal differentiation of adult hippocampal progenitors. In addition, the weight gain and food intake of mice treated with ghrelin were significantly increased when compared to

Fig. 1. Expression of GHS-R1a in adult hippocampal progenitor cells. Confocal microscopic images show that adult hippocampal progenitor cells stained with Ki-67 (red) are positive for immunoreactivity of GHS-R1a (green). 4',6-Diamidino-2-phenylindole staining revealed nuclei of dentate granule cells of hippocampus. Scale bar represents 30 μ m.

saline-treated group as expected (data not shown).

Immunoneutralization of ghrelin reduces proliferation and neuronal differentiation of adult hippocampal progenitor cells in the SGZ

Next we addressed whether endogenous ghrelin influence adult hippocampal neurogenesis using mice treated with anti-ghrelin antiserum for 8 days. According to manufacturer's instructions, rabbit anti-ghrelin antibody we used in this study showed 100% cross-reactivity with mouse ghrelin. As shown in Fig. 3, in animals treated with anti-ghrelin antibody, the number of BrdU-positive cells in the SGZ (25.5 ± 1.1 /mm) was significantly reduced when compared to mice treated with normal rabbit IgG (36.3 ± 1.5 /mm) ($p < 0.01$; Fig. 3A and C). The number of DCX-positive cells was also decreased in animals treated with neutralizing ghrelin antibody (41.5 ± 1.3 /mm vs. 50.4 ± 1.7 /mm) ($p < 0.01$; Fig. 3B and D). These results suggest that endogenous ghrelin regulates neurogenesis in the SGZ. Additionally, the weight gain and food intake of animals treated with anti-ghrelin antibody were significantly reduced when compared to normal IgG-treated group (data not shown).

Fig. 2. Effect of intraperitoneally administered ghrelin on cell proliferation and neuronal differentiation in the dentate gyrus. (A and B), Ghrelin treatment increased the number of BrdU- and doublecortin (DCX)-immunoreactive cells in the subgranular region. (C and D), Quantitative analysis indicated that the number of BrdU- and DCX-positive cells in the subgranular zone are significantly increased in ghrelin-treated mice compared with saline-treated mice. The data expressed as the mean $\pm$ SEM (n=5/group). Scale bar represents 100 μ m; * $p < 0.01$ vs. saline-treated group.

Discussion

In the current study we report for the first time that peripheral administration of ghrelin stimulates proliferation and differentiation of adult mice hippocampal neuronal progenitor cells in the SGZ. The proliferative effect of ghrelin was demonstrated by the observation that the number of BrdU-positive cells was significantly increased in animals treated with ghrelin for 8 days. Furthermore, an immunohistochemical study

using DCX indicated that the number of newly generated neurons in the SGZ increased significantly. It is evident that even endogenous ghrelin plays an important role in neurogenesis because immunoneutralization of ghrelin inhibited BrdU incorporation and decreased DCX-positive cells in the SGZ.

In the present study, GHS-R1a expression was detected in hippocampal progenitor cells, suggesting an involvement of ghrelin in adult hippocampal neurogenesis. In fact, our results suggest that ghrelin-stim-

Fig. 3. Effect of anti-ghrelin antibody on cell proliferation and neuronal differentiation in the dentate gyrus. (A and B), Immunoneutralization of ghrelin activity decreased the number of BrdU- and doublecortin (DCX)-immunoreactive cells in the subgranular region. (C and D), Quantitative analysis indicated that the number of BrdU- and DCX-positive cells in the subgranular zone are significantly reduced in mice treated with anti-ghrelin antibody when compared to normal IgG-treated mice. The data expressed as the mean $\pm$ SEM (n=5/group). Scale bar represents 100 μ m; * p < 0.01 vs. normal IgG-treated group.

ulated neurogenesis is likely to be the consequence of increasing cell proliferation. Similar neuroproliferative effects of ghrelin were observed in fetal spinal cord [8], NTS [9], DMNV [10], and cultured adult hippocampal progenitor cells [11]. Conversely, in this study the BrdU incorporation was reduced in animals passively-immunized against ghrelin. Taken together, these findings support the hypothesis that ghrelin increases neuronal proliferation in the adult central nervous system (CNS). Next we showed that early neu-

ronal differentiation was also affected by ghrelin, because DCX-positive cells were increased by exogenous administration of ghrelin and decreased by immunoneutralization of ghrelin. It has been reported that *in vitro* administration of ghrelin induces the differentiation of several type of cells, including osteoblasts, adipocytes and neurons [17]. Therefore, these observations suggest that ghrelin increases neuronal differentiation in the adult CNS. However, the phenotype of progenitor cell progeny in the DG after

long-term ghrelin treatment remains to be determined. Another stage of neurogenesis is survival of newly generated neurons and ghrelin may affect this process. This assumption is supported by other study, in which ghrelin protected hippocampal neurons from cell death induced by cerebral ischemia/reperfusion injury [18]. Similar protective effects of ghrelin were observed in hypothalamic and cortical neurons exposed to oxygen-glucose deprivation insult [15, 19]. Therefore, we assume that the observed increase in neurogenesis by ghrelin treatment is at least in part an effect on cell survival. It remains to be determined whether ghrelin is directly involved in cell survival of newly generated neurons in the DG.

Memory impairment is very common in several disorders associated with GCL pathology, including ischemic injury and epilepsy. In ischemic injury, memory impairment is caused by neuronal cell loss in the hippocampus [20]. A number of growth factors, such as fibroblast growth factor-2, insulin-like growth factor-I and vascular endothelial growth factor, improve cognitive function either by direct effects on the generation of new neurons or indirectly through neurotrophic effects that promote the survival of new neurons [21]. Another example of the relationship between memory and adult neurogenesis is epilepsy, in which hip-

pocampal neurogenesis is inhibited [22]. Recently it has been reported that ghrelin suppresses the onset time of pentylenetetrazole-induced seizures [23]. However, the precise mechanism of antiepileptic effect of ghrelin is unknown. Therefore, it is plausible that ghrelin-induced stimulation of neurogenesis in hippocampal progenitor cells may play an important role in improving cognitive functions and reducing neuronal cell loss after hippocampal damage induced by ischemic injury or epilepsy. Indeed, systemic administration of ghrelin is known to enhance learning and memory in mice [24]. Further studies are needed to confirm this issue.

In conclusion, our data show that systemic administration of ghrelin increases proliferation and early neuronal differentiation of adult hippocampal progenitor cells. Thus, ghrelin may be a potential therapeutic approach for impaired learning and memory processing.

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