Ghrelin regulates cell cycle-related gene expression in cultured hippocampal neural stem cells

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Abstract

We have previously demonstrated that ghrelin stimulates the cellular proliferation of cultured adult rat hippocampal neural stem cells (NSCs). However, little is known about the molecular mechanisms by which ghrelin regulates cell cycle progression. The purpose of this study was to investigate the potential effects of ghrelin on cell cycle regulatory molecules in cultured hippocampal NSCs. Ghrelin treatment increased proliferation assessed by CCK-8 proliferation assay. The expression levels of proliferating cell nuclear antigen and cell division control 2, well-known cell-proliferating markers, were also increased by ghrelin. Fluorescence-activated cell sorting analysis revealed that ghrelin promoted progression of cell cycle from G₀/G₁ to S phase, whereas this progression was attenuated by the pretreatment with specific inhibitors of MEK/extracellular signal-regulated kinase 1/2, phosphoinositide 3-kinase/Akt, mammalian target of rapamycin, and janus kinase 2/signal transducer and activator of transcription 3. Ghrelin-induced proliferative effect was associated with increased expression of E2F1 transcription factor in the nucleus, as determined by Western blotting and immunofluorescence. We also found that ghrelin caused an increase in protein levels of positive regulators of cell cycle, such as cyclin A and cyclin-dependent kinase (CDK) 2. Moreover, p27^{KIP1} and p57^{KIP2} protein levels were reduced when cell were exposed to ghrelin, suggesting downregulation of CDK inhibitors may contribute to proliferative effect of ghrelin. Our data suggest that ghrelin targets both cell cycle positive and negative regulators to stimulate proliferation of cultured hippocampal NSCs.

Key Words

- ▶ ghrelin
- ▶ hippocampus
- ▶ progenitor
- ▶ proliferation
- ▶ cell cycle

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Introduction

Ghrelin is a unique 28-amino acid peptide hormone mainly produced in the stomach. The peptide is known to act through the activation of the growth hormone (GH) secretagogue receptor 1a (Kojima *et al.* 1999). Earlier studies revealed that ghrelin exerts a stimulatory influence on GH release primarily at the levels of anterior pituitary and

hypothalamus and enhances food intake to regulate body weight and energy homeostasis (Date *et al.* 2000). Ghrelin also has wide variety of physiological actions throughout the body, including effects on exocrine and endocrine pancreatic functions, carbohydrate metabolism, the cardiovascular system, gastric secretion, stomach motility

and sleep (Van der Lely *et al.* 2004, Ghigo *et al.* 2005, Kojima & Kangawa 2005). In the central nervous system (CNS), ghrelin shows various nonendocrine effects to control different brain functions, such as enhancement of learning and memory (Diano *et al.* 2006), attenuation of anxiety and depression (Carlini *et al.* 2004, Lutter *et al.* 2008), modulation of reward and motivation (Naleid *et al.* 2005, Abizaid *et al.* 2006, Jiang *et al.* 2006), and protection of neuronal cells (Chung *et al.* 2007, 2008, Miao *et al.* 2007, Jiang *et al.* 2008, Hwang *et al.* 2009, Moon *et al.* 2009a, Lee *et al.* 2010a,b).

Neurogenesis is known to persist throughout adulthood in several mammalian species in the hippocampus, especially the subgranular zone of the dentate gyrus (DG) and in the subventricular zone of the lateral ventricle (Ming & Song 2005, Zhao *et al.* 2008). Hippocampus plays an important role in the process of learning, memory and emotional responses (Zhao *et al.* 2008); hence, a lot of attention has been paid on hippocampal neurogenesis. Exact understanding of the mechanisms involved in the adult hippocampal neurogenesis may augment the development of new therapy using stem-cell-based strategies against neurodegenerative diseases. We have previously reported that ghrelin regulates adult hippocampal neurogenesis *in vivo* and *in vitro*. Specifically, ghrelin administration enhances hippocampal neurogenesis in adult mice (Moon *et al.* 2009b). Our studies using ghrelin-knockout mice demonstrated that deletion of ghrelin resulted in lower numbers of progenitor cells in the DG, whereas ghrelin treatment restored progenitor cell numbers to those of wild-type controls (Li *et al.* 2013). Furthermore, we also reported that ghrelin induces cellular proliferation of cultured adult rat hippocampal neural stem cells (NSCs) via the activation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), phosphoinositide 3-kinase (PI3K)/Akt/glycogen synthase kinase (GSK)-3 β , PI3K/Akt/mammalian target of rapamycin (mTOR)/p70^{S6K} and janus kinase (JAK) 2/signal transducer, and activator of transcription (STAT) 3 signaling pathways (Chung *et al.* 2013). Collectively, these data suggest that ghrelin acts as a neurogenic agent. However, the precise mechanism of this stimulatory effect of ghrelin on hippocampal NSCs proliferation has not been fully understood.

Several cell cycle molecules and signaling pathways have been implicated in the regulation of adult hippocampal neurogenesis (Patricio *et al.* 2013). The progression of cell cycle is regulated by the activities of two important cell cycle regulators, cyclins and cyclin-dependent kinases (CDKs) (Beukelaers *et al.* 2012).

During the G₁ phase, cyclin D-CDK4/6 complexes are activated. Cyclin E and A, together with their catalytic enzyme, CDK2, are necessary for the advancement of the cell cycle from the G₁ into S phase and progression through S phase, respectively. Cyclin B-CDK1 complexes contribute to M phase entry. The transcription factor E2F1 is involved in the regulation of cell cycle regulation (Yoshikawa 2000). Additionally, the activity of cyclin-CDK complex can be regulated by the inhibitory interaction with CDK inhibitors (CKIs), such as p21^{CIP1}, p27^{KIP1} and p57^{KIP2} (Vidal & Koff 2000). Therefore, the balance between the levels of promitogenic cyclins and antimitogenic CKIs is thought to be important for the progression into S phase. Mitogenic signals, such as insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF), are known to stimulate proliferation of cortical precursors through upregulation of cyclins and downregulation of CKIs (Li & Cicco-Bloom 2004, Mairet-Coello *et al.* 2009). In contrast, pituitary adenylate cyclase-activating polypeptide exerts antimitogenic effect via augmenting expression of p57^{KIP2} (Carey *et al.* 2002). Moreover, it has been reported that ghrelin promotes cell cycle progression in intestinal epithelial cells (Waseem *et al.* 2014) and augments the expression of positive regulators of cell cycle progression in murine T-cells (Lee *et al.* 2014). Based on these findings, we hypothesized that the proliferative actions of ghrelin on hippocampal NSCs may be related to control of cell cycle molecules. At present, no data are available on the effects of ghrelin on cell cycle regulators in hippocampal NSCs. Therefore, in this study, we performed a series of experiments in order to pursue further the biological effects of ghrelin in the CNS and determine the neurogenic mechanism of action of ghrelin in adult rat hippocampal NSCs. We found that ghrelin-mediated proliferation was associated with the induction of nuclear E2F1 and regulation of other cell cycle-related genes.

Materials and methods

Materials

Rat ghrelin was obtained from Peptides International (Louisville, KY, USA). NSC expansion media, Dulbecco's modified Eagle's medium (DMEM)/F12 and B27 supplement were obtained from Gibco/Invitrogen. B-27 is an optimized serum substitute developed for low-density plating and long-term viability and growth of CNS neurons (Brewer *et al.* 1993). PD98059, U0126,

LY294002, rapamycin and cucurbitacin I were obtained from Tocris (Ellisville, MO, USA) and Akt inhibitor VIII was procured from Santa Cruz Biotechnology. All tissue culture reagents were obtained from Gibco/Invitrogen, and all other reagents were purchased from Sigma, unless otherwise indicated.

Adult rat hippocampal NSC cultures and treatments

Adult rat hippocampal NSCs were obtained from Chemicon (Catalog No. SCR022). These cells are ready-to-use primary NSCs isolated from the hippocampus of adult Fisher 344 rats. They were grown in an NSC expansion medium containing DMEM/F12 medium with L-glutamine, B27 supplement, 1× solution of penicillin, streptomycin and fungizone, and bFGF (20 ng/mL). Tissue culture plastic- or glass wares that were used to culture hippocampal NSCs were coated with poly-L-ornithine (10 µg/mL) and laminin (5 µg/mL). The hippocampal NSCs were maintained at 37°C in a 5% CO₂ humidified incubator and passaged once every 5–6 days. To determine whether ghrelin stimulates the proliferation of hippocampal NSCs, cells were treated with ghrelin (100 nM) for 24, 48, and 72 h. This dose of ghrelin (100 nM) was selected on the basis of our previous report showing that it increased the proliferation of hippocampal NSCs and it was the lowest dose with the maximum response (Chung *et al.* 2013). Experiments were also performed by incubating the cells with the following pharmacological inhibitors: 20 µM PD98059 for 0.5 h, 10 µM U0126 for 0.5 h, 20 µM LY294002 for 1 h, 100 nM Akt inhibitor VIII for 1 h, 200 nM rapamycin for 1 h or 1 nM cucurbitacin I for 0.5 h. All experiments were performed three times in duplicate.

Evaluation of cell proliferation using CCK-8 proliferation assay

Experiments for cell proliferation were performed in 24-well plates. Cells were seeded at a density of 1 × 10⁵ cells/mL of neuronal expansion media containing bFGF. After 24 h, the media were replaced with fresh media with ghrelin (100 nM) or a vehicle and incubated for 24, 48 and 72 h. The cell proliferation was determined using the CCK-8 proliferation assay (Enzo Life Sciences, Lausen, Switzerland) according to the manufacturer's instructions. In brief, 20 µL CCK-8 solution were added into each well (containing 200 µL medium), and further cultured for 2 h at 37°C. The absorbance of each group at 450 nm was detected (*n* = 3) using an absorbance microplate reader

(Molecular Devices, LLC, Sunnyvale, CA, USA) and it was directly proportional to the number of living cells.

Fluorescence-activated cell sorting (FACS) analysis

Cell cycle distribution was examined by FACS analysis. 1 × 10⁶ cells were collected and fixed with 3.7% paraformaldehyde. After fixation, the propidium iodide (50 µg/mL)-staining solution with RNase A (BD Biosciences, San Jose, CA, USA) was added. After 30-min incubation, the cells were filtered on a nylon mesh filter. Stained samples were analyzed by flow cytometric analysis using FACSCalibur (BD Biosciences, San Jose, CA, USA).

Real-time RT-PCR

Total RNA from hippocampal NSCs was extracted using the Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and was reverse transcribed using the Superscript II reverse transcriptase (Life Technologies) at 42°C with random hexamer priming. An RNA control tube containing all reverse transcription reagents except reverse transcriptase was included as a negative control to monitor genomic DNA contamination. Real-time RT-PCR was used to determine mRNA levels of proliferating cell nuclear antigen (PCNA) and cell division control (CDC) 2 in hippocampal NSCs using primers specific for PCNA and CDC2 (The Rat Cell Cycle RT² Profiler PCR Array, Qiagen), and β-actin (sense: 5'-ATG GGT CAG AAG GAC TCC TAC G-3' and antisense: 5'-AGT GGT ACG ACC AGA GGC ATA C-3'). Reactions were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems).

Western blotting

Cells 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 Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF and 10 µg/mL aprotinin. Cell lysates were separated by 12% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad). For the detection of E2F1, cells were fractionated into nuclear and cytoplasmic contents using the Nuclear/Cytoplasmic Extraction Kit (Pierce Biotechnology) according to the manufacturer's instructions. The membranes were soaked in a blocking buffer (1× Tris-buffered saline (TBS), 1% bovine serum albumin (BSA) and 1% nonfat dry milk) for 1 h and incubated overnight at 4°C with the primary

Figure 1

Ghrelin stimulates proliferation of adult rat hippocampal NSCs. (A) Cells were treated with ghrelin (100 nM) for 24, 48 and 72 h, and the cell proliferation was assayed using the CCK-8 proliferation assay. (B) Cells were treated with ghrelin (100 nM) for 24 h. Protein lysates were prepared and assayed by Western blotting using specific anti-PCNA and anti-CDC2 antibodies. The band intensities of PCNA and CDC2 were normalized to the band intensity of β -actin. (C) Time course of ghrelin-induced effects on PCNA and CDC2 mRNA levels. Cells were treated with ghrelin (100 nM) for 4, 8, 12 and 24 h. Real-time RT-PCR was performed to measure mRNA levels of PCNA and CDC2 and adjusted by β -actin. The data are expressed as percentage of vehicle and are shown as the mean $\pm$ S.E.M. of three different experiments (each experiment was performed in duplicate). * $P < 0.05$ vs vehicle-treated control.

antibodies against PCNA, CDC2, Laminin A, GAPDH (Cell Signaling; 1:1000), and CDK2, cyclin A, p27^{KIP1} and p57^{KIP2}, E2F1 (Santa Cruz Biotechnology; 1:1000). Blots were developed using a peroxidase-conjugated antirabbit 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).

Immunocytochemistry

Adult rat hippocampal NSCs were fixed in 4% paraformaldehyde for 20 min at room temperature. Cells

were washed twice with phosphate-buffered saline (PBS) and permeabilized with PBS containing 0.4% Triton X-100 for 20 min, followed by blocking with TBS containing 0.02% Tween 20 and 10% normal goat serum for 1 h at room temperature. Primary antibody incubations were performed at 4°C overnight in TBS containing 0.02% Tween 20 and 3% BSA. Cells were incubated in a blocking solution (0.3% Triton X-100, 1% BSA and 3% normal goat serum in PBS) for 2 h. After overnight incubation with primary antibody against E2F1 (1:400; Santa Cruz Biotechnology) at 4°C, cells were rinsed with PBS, followed by incubation with a secondary antibody (Alexa Fluor 488 goat antirabbit IgG, 1:400, Invitrogen) for 4 h at

Figure 2

Fluorescence-activated cell sorting (FACS) analysis of cell cycle distribution. (A) Following 16-h starvation and pretreatment with 20 μ M PD98059 for 0.5 h, 10 μ M U0126 for 0.5 h, 20 μ M LY294002 for 1 h, 100 nM Akt inhibitor VIII for 1 h, 200 nM rapamycin for 1 h or 1 nM cucurbitacin I for 0.5 h, cells were treated with ghrelin (100 nM) for 8 h. The cells were harvested by propidium iodide (50 μ g/mL)-staining solution and subjected to FACS analysis. Results were expressed as percentage of total cells in G₀/G₁, S or G₂/M phase of the cell cycle. Values represent the mean $\pm$ S.E.M. of three different experiments (each experiment was performed in duplicate). (B) The bar graphs represent the S phase ratio expressed as the percentage of vehicle. * P < 0.05 vs vehicle-treated cells, † P < 0.05 vs ghrelin-treated cells.

Figure 3

Ghrelin increases the nuclear expression of transcription factor E2F1. Cells were treated with ghrelin (100 nM) for 4, 8, 12 and 24 h. Nuclear and cytoplasmic lysates were prepared and assayed by Western blotting using specific anti-E2F1, anti-laminin A and anti-GAPDH antibodies. (A) The nuclear E2F1 band intensity was normalized to the laminin A band intensity. (B) The cytoplasmic E2F1 band intensity was normalized to the GAPDH band intensity. Representative Western blotting images are shown in the upper insets. The data are expressed as the mean $\pm$ S.E.M. of three different experiments (each experiment was performed in duplicate). (C) The bar graphs represent the nuclear/cytoplasmic E2F1 ratio expressed as the percentage of control. * P < 0.05 vs control.

Figure 4

Confocal microscopic analysis of E2F1 expression. Cells were treated with ghrelin (100 nM) for 4, 8, 12 and 24 h. Cells were incubated with primary antibody to E2F1. Cells were counterstained with DAPI, and images were captured using confocal microscopy; scale bar, 5 μ m.

room temperature. Images were acquired by the Carl Zeiss LSM 700 Meta confocal microscope and analyzed using Carl Zeiss ZEN image software.

Statistical analysis

Data are presented as mean $\pm$ S.E.M. of three different experiments (each experiment was performed in duplicate). Statistical analysis between groups was performed using Student's *t*-test for the comparison of two means or one-way ANOVA and the 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.

Results

Ghrelin stimulates proliferation of adult rat hippocampal NSCs

Based on our previous results demonstrating that ghrelin increases BrdU incorporation of cultured adult

Figure 5

Ghrelin increases the expression of cyclin A and CDK2. (A and B) Cells were treated with ghrelin (100 nM) for 4, 8, 12 and 24 h. (C and D) Cells were preincubated with 20 μ M PD98059 for 0.5 h, 20 μ M LY294002 for 1 h, 200 nM rapamycin for 1 h or 1 nM cucurbitacin I for 0.5 h and then treated with ghrelin (100 nM) for 8 h. Lysates were prepared and assayed by Western blotting using specific anti-cyclin A, anti-CDK2 and anti- β -actin antibodies. Cyclin A (A and C) and CDK2 (B and D) band intensities were normalized to the β -actin band intensity and expressed as relative band intensity. Representative Western blotting images are shown in the upper insets. The data are expressed as the mean $\pm$ S.E.M. of three different experiments (each experiment was performed in duplicate). * P < 0.05 vs control; † P < 0.05 vs ghrelin-treated cells.

rat hippocampal NSCs (Chung *et al.* 2013), CCK-8 proliferation assay was used to confirm the effect of ghrelin on cell proliferative ability. As shown in Fig. 1A, ghrelin (100 nM) significantly increased cell proliferation in a time-dependent manner. Next, we further analyzed well-defined cell-proliferating markers, PCNA and CDC2, by Western blotting. After 24-h treatment with ghrelin (100 nM), protein levels of PCNA and CDC2 were significantly increased by 126.3 ± 7.9 and $139.0 \pm 7.8\%$, respectively (P < 0.05 vs vehicle-treated control), as shown in Fig. 1B. These results indicate that ghrelin increased the expression of well-known cell cycle-related genes, PCNA and CDC2, which are essential for progression through mitosis. Ghrelin also promoted the levels of mRNA for PCNA and CDC2 assessed by real-time RT-PCR (Fig. 1C).

Multiple pathways are involved in ghrelin-induced cell cycle progression of adult rat hippocampal NSCs

Our previous data indicated that pretreatment of cells with specific inhibitors of MEK/ERK1/2, PI3K/Akt, mTOR, and JAK2/STAT3 attenuated ghrelin-induced hippocampal NSCs proliferation (Chung *et al.* 2013). To further investigate ghrelin-induced cell proliferation, we analyzed the effect of ghrelin on cell cycle entry using FACS analysis of distribution of cells in each phase of the cell cycle. Following growth factor deprivation for 16 h, cells were treated with vehicle or ghrelin (100 nM) for 8 h. In vehicle-treated cells, $84.4 \pm 0.2\%$ of cells were in G_0/G_1 ,

$6.0 \pm 0.6\%$ were in S and $9.7 \pm 0.6\%$ were in G_2/M phase (Fig. 2A). When cells were incubated with ghrelin for 8 h, the G_0/G_1 population was decreased to $80.7 \pm 0.5\%$, the S phase population was increased to $10.9 \pm 0.8\%$ and the G_2/M population was decreased to $8.4 \pm 0.7\%$ (Fig. 2A). These results indicated that hippocampal NSCs entered the S phase after 8-h treatment with ghrelin. However, when cells were exposed to MEK1 inhibitor PD98059, MEK1/2 inhibitor U0126, PI3K inhibitor LY294002, Akt inhibitor VIII, mTOR inhibitor rapamycin or JAK2/STAT3 inhibitor cucurbitacin I, ghrelin-induced increase in S phase ratio was significantly attenuated (Fig. 2B).

Ghrelin increases nuclear expression of transcription factor E2F1 in adult rat hippocampal NSCs

It has been demonstrated that the transcription factor E2F1 is involved in controlling proliferation and neuronal cell numbers in the postnatal and adult brain (Cooper-Kuhn *et al.* 2002). To identify changes in subcellular localization of E2F1, Western blotting was conducted to detect E2F1 in nuclear and cytoplasmic fractions. Figure 3 shows ghrelin-induced increase in E2F1 protein both in the nuclear (Fig. 3A) and cytoplasmic (Fig. 3B) fractions of cells. We also calculated the nuclear/cytoplasmic E2F1 ratio and found that ghrelin significantly increased the ratio after 8-h treatment (Fig. 3C), suggesting increased expression of E2F1 in the nucleus. These results were confirmed by immunofluorescence confocal microscopy,

Figure 6

Ghrelin decreases the expression of p27^{KIP1} and p57^{KIP2}. (A and B) Cells were treated with ghrelin (100 nM) for 4, 8, 12 and 24 h. (C and D) Cells were preincubated with 20 μ M PD98059 for 0.5 h, 20 μ M LY294002 for 1 h, 200 nM rapamycin for 1 h or 1 nM cucurbitacin I for 0.5 h and then treated with ghrelin (100 nM) for 8 h. Lysates were prepared and assayed by Western blotting using specific anti-p27^{KIP1}, anti-p57^{KIP2} and anti- β -actin antibodies. p27^{KIP1} (A and C) and p57^{KIP2} (B and D) band intensities were normalized to the β -actin band intensity and expressed as relative band intensity. Representative Western blotting images are shown in the upper insets. The data are expressed as the mean $\pm$ s.e.m. of three different experiments (each experiment was performed in duplicate). * $P < 0.05$ vs control; † $P < 0.05$ vs ghrelin-treated cells.

which showed an increased staining pattern when cells were treated with ghrelin (Fig. 4).

Ghrelin increases the expression of cyclin A and CDK2 in adult rat hippocampal NSCs

To determine the mechanisms involved in ghrelin-induced proliferation of hippocampal NSCs, we examined the expression of cyclin A, which is accumulated during S phase (Yam *et al.* 2002). Regulation of expression of cyclin A is mediated by the transcription factor E2F1 (Schulze *et al.* 1995). As shown in Fig. 5A, ghrelin increased cyclin A protein levels in a time-dependent manner, with a significant rise after 8-h treatment. Similar increase in protein levels of CDK2 was also observed after 4-h ghrelin treatment (Fig. 5B). We further determined which signaling pathways are involved in ghrelin-induced increase in cyclin A and CDK2 expression. We found that pretreatment of cells with PD98059, LY294002, rapamycin or cucurbitacin I significantly attenuated ghrelin-mediated enhancement of cyclin A and CDK2 protein levels (Fig. 5C and D).

Ghrelin decreases the expression of p27^{KIP1} and p57^{KIP2} in adult rat hippocampal NSCs

We also examined the expression of two negative regulators of cell cycle, p27^{KIP1} and p57^{KIP2}. We first analyzed the expression of p27^{KIP1}, because it is likely an important CKI that induces cell cycle exit in proliferative cells (Cobrinik

2005). As shown in Fig. 6A, ghrelin treatment significantly decreased p27^{KIP1} protein levels by 65% at 4 h, 70% at 8 h and 58% at 12 h. The protein levels of p27^{KIP1} remained reduced even 24 h after ghrelin treatment, exhibiting a 47% decrease. We also examined the effect of ghrelin on the expression of p57^{KIP2}. Ghrelin elicited a significant decrease in p57^{KIP2} protein levels (from 75 to 62%) at 4, 8, 12 and 24 h (Fig. 6B). Next, the pathways mediating the ghrelin-induced decrease in p27^{KIP1} and p57^{KIP2} expression were examined by exposure of cells to PD98059, LY294002, rapamycin or cucurbitacin I. As shown in Fig. 6C and D, these inhibitors attenuated the decrease in p27^{KIP1} and p57^{KIP2} protein levels of cells subjected to ghrelin treatment.

Discussion

In the current study, our data indicate that ghrelin-induced increase in nuclear expression of E2F1 is associated with ghrelin-mediated promotion of cell cycle progression in cultured adult rat hippocampal NSCs. The proliferative effect of ghrelin appears to be mediated by increased expression of genes that promote progression through the cell cycle, such as cyclin A and CDK2, while inhibiting expression of genes that trigger cell cycle exit, such as p27^{KIP1} and p57^{KIP2}. Ghrelin-induced cell cycle progression from G₀/G₁ to S phase was dependent on the activities of MEK/ERK1/2, PI3K/Akt/mTOR and JAK2/STAT3 signaling pathways.

In previous paper, we found that ghrelin increased the proliferation of cultured hippocampal NSCs assessed by BrdU incorporation, suggesting mitogenic potential of ghrelin (Chung *et al.* 2013). In this study, the proliferative effect of ghrelin has been confirmed by CCK-8 proliferation assay and enhancement of expression of PCNA and CDC2, well-defined and commonly used cell proliferation markers (Castedo *et al.* 2002, Moldovan *et al.* 2007). PCNA, a marker for G₁ and S phase, acts as a homotrimer and helps increase the processivity of leading strand synthesis during DNA replication (Moldovan *et al.* 2007). CDC2, also called cyclin-dependent kinase 1, has to be active to allow cells to progress from the G₂ to the M phase (Castedo *et al.* 2002). In the current study, 24-h incubation of cells with ghrelin significantly increased the expression of PCNA and CDC2, suggesting that ghrelin augmented entry into and progression of hippocampal NSCs through the cell cycle.

To investigate the ability of ghrelin to cause hippocampal NSCs entry into the cell cycle, we performed FACS analysis of cell distribution in each phase of the cell cycle. We found that ghrelin increased the number of cells in S phase with a decrease in number of cells in G₀/G₁ phase, indicating ghrelin-mediated promotion of entry into S phase. Multiple signaling pathways and molecules are involved in regulation of the proliferation of neural progenitor cells. For example, a dominant negative Akt inhibits cell proliferation (Peltier *et al.* 2007) and the activation of ERK is important for the proliferation of neural progenitor cells (Learish *et al.* 2000, Zhou *et al.* 2004). Inhibitor of GSK-3 β , a downstream effector of Akt, promotes the proliferation of progenitor cells, while β -catenin overexpression increases the proliferation (Adachi *et al.* 2007). The activation of mTOR/p70^{S6K} signaling has been reported to increase the proliferation of NSCs *in vitro* (Ryu *et al.* 2003). In addition, STAT3 inhibition blocked leptin-induced proliferation of adult hippocampal neural progenitor cells (Garza *et al.* 2008). These findings were supported by our previous report, where we showed that ghrelin increased the phosphorylation of ERK1/2, Akt, STAT3, GSK-3 β , mTOR and p70^{S6K}, and chemical inhibition of MEK/ERK1/2, PI3K/Akt, mTOR and STAT3 reversed ghrelin-induced increase in the proliferation of hippocampal NSCs (Chung *et al.* 2013). In agreement with these observations, we found that ghrelin-induced progression into S phase in hippocampal NSCs was attenuated when cells were pretreated with specific inhibitors of MEK/ERK1/2, PI3K/Akt, mTOR and

JAK2/STAT3. Taken together, our previous and current results suggest that multiple signaling pathways, such as MEK/ERK1/2, PI3K/Akt/GSK-3 β , PI3K/Akt/mTOR/p70^{S6K} and JAK2/STAT3, are involved in ghrelin-induced cell cycle progression and proliferation of hippocampal NSCs.

In this study, we have shown that ghrelin increases the nuclear expression of E2F1, which is requisite for enabling E2F1 to reach target DNA sequences (Muller & Helin 2000). Considering that E2F1 expression is essential in adult neurogenesis (Cooper-Kuhn *et al.* 2002), the increase in expression of nuclear E2F1 may contribute to ghrelin-mediated proliferation of hippocampal NSCs. The E2F1 transcription factor is known to bind exclusively to retinoblastoma (Rb) protein in a cell cycle-dependent manner (Yoshikawa 2000). During the G₁ phase, the cyclin D/CDK4-6 complex phosphorylates the Rb protein, resulting in sequential phosphorylation by cyclin E/CDK2 and the release of free E2F1 (Abrous *et al.* 2005). CDK2 then combines with cyclin A to maintain the cells in S phase (Beukelaers *et al.* 2012). Indeed, cyclin A, a marker of S phase, was elevated by ghrelin and this increase was paralleled by enhanced protein levels of CDK2. Moreover, exposure of cells to PD98059, LY294002, rapamycin or cucurbitacin I resulted in significant attenuation of the stimulatory effect of ghrelin on cyclin A and CDK2 protein levels, indicating that this peptide increased the expression of cyclin A and CDK2 through the activation of MEK/ERK1/2, PI3K/Akt/mTOR and JAK2/STAT3 pathways. Therefore, we propose that mitotic ghrelin signaling increases cyclin A levels by stimulating nuclear expression of E2F1, leading to formation of active cyclin A/CDK2 complexes and further hyperphosphorylation of Rb. Increased activity of cyclin A/CDK2 triggers the initiation of DNA replication and thus entry into the S phase of the cell cycle.

Our results suggest that, besides stimulating promitogenic cyclin A and CDK2, ghrelin also induces downregulation of cell cycle inhibitors, p27^{KIP1} and p57^{KIP2}, to promote S phase entry of hippocampal NSCs. Our data also suggest that the activation of MEK/ERK1/2, PI3K/Akt/mTOR and JAK2/STAT3 is involved in the regulation by ghrelin in p27^{KIP1} and p57^{KIP2} expression. The antimitogenic molecules p27^{KIP1} and p57^{KIP2} negatively regulate the progression of cell cycle by binding and inhibiting complexes of cyclin E/CDK2 and cyclin A/CDK2 and thus promote cell cycle exit in proliferative cells (Vidal & Koff 2000, Abrous *et al.* 2005). Reduced levels of p27^{KIP1} and p57^{KIP2} have been implicated in the mechanism of IGF-1- or bFGF-mediated proliferative

action in cortical precursor cells (Li & Cicco-Bloom 2004, Mairet-Coello *et al.* 2009). Similar findings were also observed in IGF-1- and FGF-2-stimulated oligodendrocyte progenitor cells (Frederick & Wood 2004). In the current study, we have shown that ghrelin downregulates p27^{KIP1} and p57^{KIP2}, helping to trigger entry into S phase of hippocampal NSCs. It is likely that ghrelin mitigates inhibitory influence on cyclin/CDK activity by the reduced expression of p27^{KIP1} and p57^{KIP2}.

Combining the current results and previous studies, ghrelin seems to be a plausible candidate that improves hippocampus-dependent cognitive function. The supporting evidences are as follows. (1) Adult neurogenesis in the DG has been proposed to mediate hippocampus-dependent learning and memory (Zhao *et al.* 2008). (2) We and others have previously reported that ghrelin-deleted mice showed impairments in memory performance, which was rescued by exogenous peripheral administration of ghrelin (Diano *et al.* 2006, Li *et al.* 2013). (3) In calorie-restricted animals, where plasma ghrelin levels are elevated (Lutter *et al.* 2008), survival of newborn cells in the DG has been increased in wild-type mice (Lee *et al.* 2002), but not in ghrelin-knockout mice (Kim *et al.* 2015) and enhanced memory performance in hippocampus-dependent behavioral test was observed (Fontan-Lozano *et al.* 2007, Adams *et al.* 2008).

In conclusion, our observations indicate that ghrelin has a mitogenic effect in cultured hippocampal NSCs. Ghrelin-induced mitogenic regulation may be mediated via enhanced progression from G₁ to S phase due to enhanced nuclear expression of E2F1 and opposite regulation of positive and negative cell cycle regulators. Therefore, ghrelin may be a possible beneficial approach for the impairments in learning and memory performance observed in various neurodegenerative diseases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Hyunju Chung: carried out the laboratory experiments, collected the data and wrote the manuscript. Seungjoon Park: designed the study, analyzed and interpreted the data and wrote the manuscript.

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