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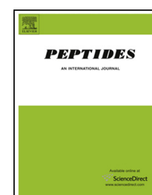

# Ghrelin suppresses proliferation of fetal neural progenitor cells, and induces their differentiation into neurons

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ABSTRACT

Although considerable progress has been made in understanding how the temporal and regional control of neural progenitor cells (NPCs) dictates their fate, their key regulators during neural development are still unknown. Ghrelin, which is isolated from porcine stomach extract, is an endogenous ligand for the growth hormone secretagogue receptor (GHS-R). The widespread expression of ghrelin and GHS-R in the central nervous system during development suggests that ghrelin may be involved in developmental neural growth. However, its role in regulating fetal NPCs is still unclear. In this study, we investigated the effects of ghrelin on primary cultured NPCs derived from fetal mouse telencephalon. The expressions of both ghrelin and its receptor were observed in NPCs using RT-PCR, immunoblotting and immunocytostaining. Interestingly, the exposure of fetal NPCs to ghrelin at concentrations of $10^{-7}$ and $10^{-9}$ M suppressed their proliferation, and caused them to differentiate into neurons and to extend neurites. These results strongly suggest that ghrelin plays an autocrine modulatory role in fetal neural development.

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## Introduction

Neural progenitor cells (NPCs) are characterized by their capacity for self-renewal and their ability to generate the major cell types of the central nervous system (CNS) [8]. NPCs undergo proliferation, migration and differentiation in the embryonic and newborn CNS, as well as in restricted regions of the adult mammalian CNS, including the subventricular zone (SVZ) and the dentate gyrus of the hippocampus [38]. Considerable progress has been made in elucidating how the temporal and regional control of NPCs contributes to cell fate decisions; however, the key regulators of NPCs and their roles in neural development are still unknown [9]. Recent studies have shown that the fate of NPCs is determined by neuropeptides such as neuropeptide Y (NPY), vasoactive intestinal peptide and galanin [29,40]. We have also demonstrated that pituitary adenylate cyclase activating polypeptide (PACAP) induces the differentiation of fetal NPCs into astrocytes via the PKC pathway [26,35,36]. Although it has been clarified that cell-intrinsic and -extrinsic factors such as neuropeptides are implicated in the process of NPC differentiation, the detailed molecular mechanisms remain unclear.

Ghrelin, which is isolated from porcine stomach extract, is an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) [19]. Although ghrelin is well known to act as a modulator of feeding behavior and energy metabolism [15–17,19,23,27,31], recent studies have suggested that it is also involved in memory functions [4] and fetal development [18,25]. In rodents, high levels of ghrelin mRNA have been detected in fetuses at embryonic day (E) 12, and E17 fetuses contain significant levels of ghrelin in their blood [25]. GHS-R mRNA has been reported in the brain and spinal cord as early as E12 and continues to be expressed in these tissues in adulthood [25,30]. The widespread expression of ghrelin and its receptor in the CNS during development suggests that ghrelin may be involved in developmental neural growth.

Recently, some groups reported that an abnormality in ghrelin expression is associated with developmental disorders such as autism and intellectual disability [1,7,10–12]. These data suggest that ghrelin is required for normal development and plays a crucial role in the regulation of NPCs. Although one group has reported that ghrelin induces proliferation of NPCs in the adult hippocampus and SVZ [3,20,21], our knowledge regarding the activity of ghrelin

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in neural development is limited. Here, we examined the effects of ghrelin in the proliferation and differentiation of fetal NPCs, revealing that, unlike in the adult brain, ghrelin suppresses proliferation and induces neural differentiation.

## Materials and methods

### *Cell culture*

Primary cell culture of NPCs was essentially carried out as previously described [34]. As previous report showed that GHS-R strongly expressed in telencephalon during CNS development [25], we performed the well-established and currently widely used method for expanding neural precursor cells from telencephalon. Briefly, telencephalons were prepared from male and female embryos (E14.5) of ICR mice by dissecting narrow region between amygdala and lateral hypothalamus. After removing dura, tissues were gently dissociated by pipetting in Hank's Balanced Salt Solution. The dissociated cells, which included NPCs, were seeded onto a culture dish previously coated with 15 μg/ml poly-L-ornithine and 1 μg/ml fibronectin (Sigma–Aldrich, St. Louis, MO). The cells were cultured in DMEM/F12 medium (Life Technologies, Carlsbad, CA) containing 25 μg/ml insulin, 100 μg/ml human apotransferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite, antibiotic antimycotic solution (Sigma–Aldrich) and 10 ng/ml recombinant human basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN) for 4 days. After being passaged at a density of $5.0 \times 10^4$ cells/cm$^2$, cells were exposed to various concentrations of ghrelin in the presence of bFGF. For proliferation assay, NPCs were cultured with 3 μg/ml BrdU (Sigma–Aldrich) for 4 days. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Showa University.

### *RT-PCR*

Total RNA was isolated from mouse embryonic NPCs and adult cerebral cortex using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands). The isolated total RNA (1 μg) was subjected to reverse transcription with Superscript III (Life Technologies), after which 1 μl of the reaction mixture was subjected to PCR. The primers used were 5′-primer, 5′-TTCCAGTTTGTCAGCGAGAGC-3′ and 3′-primer 5′-GAGAATGGGGTTGATGGCAG-3′ for GHS-R and 5′-primer 5′-AGAGAAAGGAATCCAAGAAGCC-3′ and 3′-primer 5′-TGGTAGGAGAGTGCTGGGAG-3′ for ghrelin. PCR was carried out with ExTaq kit (Takara, Shiga, Japan). Both primers were designed to pinch introns. For amplification, an initial denaturation step of 3 min at 95 °C was followed by 30 cycles of 30 s at 95 °C, 30 s at 60 °C, and 45 s at 72 °C, with the last cycle being extended for 7 min at 72 °C. PCR products were visualized by staining with ethidium bromide after electrophoresis on a 1% agarose gel.

### *Immunoblot analysis*

NPCs washed with PBS were lyzed with 10 volumes of lysis buffer containing 10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of aprotinin, pepstatin A, antipain, leupeptin, and 50 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 0.15 M NaCl and 1% Triton X-100 (Sigma–Aldrich). The lysate was centrifuged at $13{,}000 \times g$ for 10 min at 4 °C and the supernatant was further centrifuged at $75{,}000 \times g$ for 20 min at 4 °C. After the concentration of protein in the clear supernatant was quantified with BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL), samples containing 20 μg of protein were subjected to sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis. The proteins were transferred onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA), which was treated with Blocking Ace (Megmilk Snow Brand Co., Ltd, Tokyo, Japan) for 1 h at room temperature. The membrane was then reacted with rabbit polyclonal antibody against GHS-R (kindly provided by Dr. M. Nakazato, Miyazaki University, Japan) overnight at 4 °C, washed with 0.2% Tween 20/Tris-buffered saline, and the protein band visualized using a secondary antibody (HRP-conjugated anti-rabbit IgG, 1:1000, Thermo Scientific Pierce) and an enhanced chemiluminescence detection system (ECL, GE Healthcare Bio-Sciences, Pittsburgh, PA).

### *Analysis of cell number*

The number of live cells was determined after 5 days of culture using a live-dead cell staining kit (BioVision Research Products, Milpitas, CA) as previously described [33]. The number of live cells were counted with an image analysis system (Image J, NIMH, Bethesda, MD) and averaged four 500 μm × 500 μm region from each well.

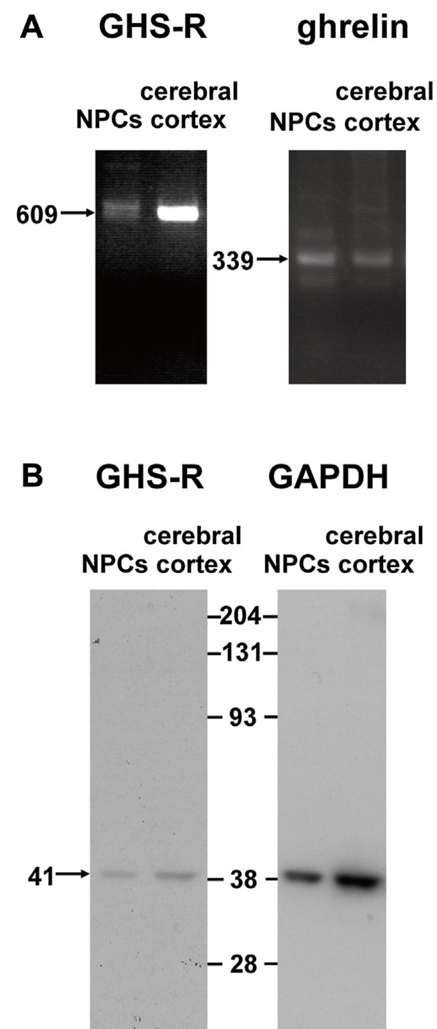


**Fig. 1.** Gene and protein expression analyses of GHS-R and ghrelin by RT-PCR and immunoblotting. (A) RT-PCR and (B) immunoblotting were carried out using 1 μg of total RNA and 20 μg of cell lysate protein prepared from neural progenitor cells (NPCs) and adult cerebral cortex as a positive control. The predicted band sizes of GHS-R and ghrelin are 609 bp and 339 bp, respectively. Both molecular weights of GHS-R and GAPDH are 41 kDa.

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