



## Ghrelin accelerates growth of olfactory ensheathing cells through ERK pathway

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### ABSTRACT

Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor (GHSR), stimulates growth hormone (GH) secretion and has both orexigenic and adipogenic effects. This study sought to understand the potential involvement of MAPKs in ghrelin-induced growth of OECs. Different concentrations of ghrelin were applied to cultured OECs to observe the growth rate of cells and changes in phosphorylation state of MAPKs ERK1/2, JNK and p38. Ghrelin-induced OECs growth was promoted primarily by phosphorylated ERK1/2, and that this phosphorylation, as well as p90rsk phosphorylation, was mediated by the ghrelin receptor GHSR. In conclusion, our study suggested that ghrelin promotes the growth of OECs primarily through the ERK pathway.

**Key words:** Ghrelin, Growth, MAPKs, OECs

Olfactory ensheathing cells (OEC) are increasingly regarded as a promising tool for repair of injured nervous system (Gunda *et al.* 2014). Found in the central nervous system, in the outer layers of the olfactory bulb, as well as in the peripheral nervous system, within the olfactory mucosa, they represent a unique glial cell type (Gao *et al.* 2015). OECs play an important role in growth, guidance, and unique regenerative capabilities of primary olfactory axons. These properties inspired studies on the potential of OECs to stimulate axon regeneration in CNS injuries. Currently, there is great interest in the possibility that OECs may have enormous potential for use in treatment of axonal injuries and demyelinating disease (Gong *et al.* 2014). Presently, little is known about the extracellular and intracellular factors that regulate OEC growth.

Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor (GHSR), is a 28-amino acid peptide produced from a 117-amino acid preprohormone. The mature form of ghrelin may undergo several post-translational modifications, including the addition of a fatty acid chain (n-octanoic acid) to the serine residue at position 3 (Kojima and Kangawa 2010). Phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) signaling pathways are activated by a variety of extracellular stimuli and regulate a wide range of cellular processes, including cell motility, survival, proliferation, and cell cycle progression. Recent studies showed that the activation of PI3K/AKT is

involved in cell survival and axonal outgrowth in neurons (Kanteti *et al.* 2014, Liang *et al.* 2014, Wang *et al.* 2014, Yu *et al.* 2014).

Several researches showed that the ghrelin can promote OEC growth. However, how to access the OEC by ghrelin was known little. In this study, we used ghrelin to affect the OEC, and find the pathway for promoting the growth of OEC.

### MATERIALS AND METHODS

*OEC culture and purification:* Rats, 1- to 3-day-old, were killed by decapitation. The olfactory bulbs were dissected and placed in Leibovitz's L-15 medium. The olfactory nerve fiber layer, together with the meningeal coverings and accompanying minute capillaries, was peeled away from the rest of the bulb with fine forceps. Pieces of olfactory nerve fiber layer were incubated with 0.1% trypsin and 0.03% collagenase at 37°C for 15 min with shaking. Trypsinization was stopped by adding DMEM containing 10% heat-inactivated fetal bovine serum. Cells were layered on 100 mm culture dishes coated with 50 µg/ml poly-D-lysine and maintained in medium. The dishes were incubated at 37°C, 5% CO<sub>2</sub>, and the medium was changed every 2 days. OECs, 5–7 days after plating, were separated from other cell types in primary cultures by immunoaffinity using antisera against p75. Cells from primary cultures were detached with 0.05% trypsin and 0.02% EDTA, centrifuged, and washed twice with medium. Cells were suspended in L-15 medium and plated on 100 mm Petri-dishes that were incubated with 1:100 goat antimouse antisera overnight at 4°C and then washed with L-15 medium. Dishes were incubated with supernatant of cultured 192 hybridoma cells

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containing p75 antibody at a 1:5 dilution in L-15 medium with 5% FBS for 2 h at 4°C. Dishes were washed with L-15, and 10 ml suspension containing cells from olfactory bulb primary cultures were plated on the antibody-treated dishes at a density of  $1 \times 10^6$  cells/dish for 30 min at 4°C. To remove unbound cells, dishes were washed 5 times with L-15 medium. Bound cells were detached using a cell scraper, centrifuged, and resuspended in medium. Cells were seeded onto poly-dlysine or laminin-coated 12 mm glass coverslips at a density of  $1 \times 10^4$  cells and were incubated at 37°C in 5% CO<sub>2</sub>. After 4 days, immunostaining revealed that 90% of cells were p75-positive, which is characteristic of OECs.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) assay:** Cells were grown in 96-well plates ( $1 \times 10^3$  cells/well) supplemented with ghrelin. Control cells were switched from RPMI-1640 to DMEM containing 0.1% dimethyl sulfoxide (DMSO). To 500, 600, 700 and 800 ng/ml ghrelin, MTT (20  $\mu$ L) was added after 24, 48 and 72 h, to each well to a final concentration of 0.5%. After a 4 h incubation at 37°C in the dark, 150  $\mu$ L DMSO was added to each well for 10 min to dissolve the formazan crystals. The absorbance was measured using a microplate reader at 490 nm. All experiments were repeated 3 times. The viability of the MGF treated cells was expressed as percentage of population growth plus the standard error of the mean (SEM) relative to that of untransfected control cells. Cell growth was calculated as follows: % growth = (mean experimental absorbance - mean control absorbance / mean control absorbance)  $\times$  100.

**Immunofluorescence:** OEC were fixed in 3.7% paraformaldehyde for 30 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS for 15 min, followed by blocking overnight at 4°C in 1% BSA in PBS with 10% goat serum. The samples were then stained with primary antibody in PBS. The primary antibody binding was detected with an Alexa Fluor 488 goat anti-rabbit IgG (H+L) secondary antibody. Images were captured with a confocal microscope. Experiments were performed in triplicate.

**Western blot:** The protein homogenates from OECs were separated using electrophoresis on 8–12% sodium dodecyl sulphate/polyacrylamide gels and transferred to immunoblot nitrocellulose membranes. Membranes were blocked for 30 min at room temperature in PBS buffer containing 5% fat-free milk and 0.1% Tween 20. Membranes were then incubated with primary antibody for at least 1 h at room temperature or overnight at 4°C. The membranes were subsequently washed 3 times with PBS containing 0.1% Tween 20, incubated with peroxidase-conjugated secondary antibodies and developed using ECL reagents.

**Statistical analysis:** Statistically significant differences between gene expression levels were determined using one-way ANOVA followed by a Newman-Keuls test with GraphPad Prism 5 software. Replicates were included in the statistical model. Differences were considered statistically significant at the 95% confidence level ( $P < 0.05$ ). Data are presented as mean  $\pm$  S D.

## RESULTS AND DISCUSSION

**OECs identified:** To identify the OECs, we used antibodies raised against p75NTR and GFAP. Double immunofluorescence combining polyclonal (anti-GFAP) and monoclonal (anti-p75NTR) antibodies was performed on cell cultures (Fig. 1).

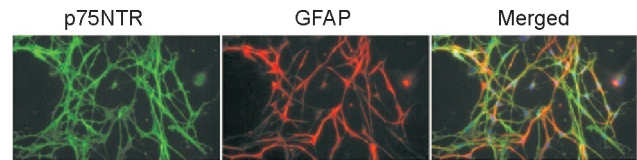


Fig. 1. Identification of rat OECs by immunofluorescence. anti-p75NTR antibody (green) and either an anti-GFAP antibody (red).

**Expression of the ghrelin receptor GHSR in OECs:** The expression of the ghrelin receptor GHSR in OECs was investigated using immunocytochemistry. The expression of the GHSR protein in OECs is given in Fig. 2.

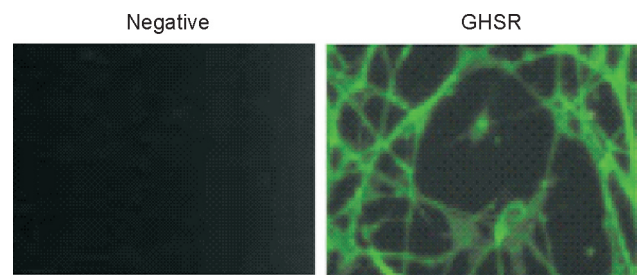


Fig. 2. Expression of the ghrelin receptor GHSR in OECs. Negative (without any antibody).

**Determination of the optimal ghrelin treatment of OECs:** The growth and survival of OECs is given in Fig. 3. Ghrelin @ 600 ng/ml for 48 h gave the maximal growth rate.

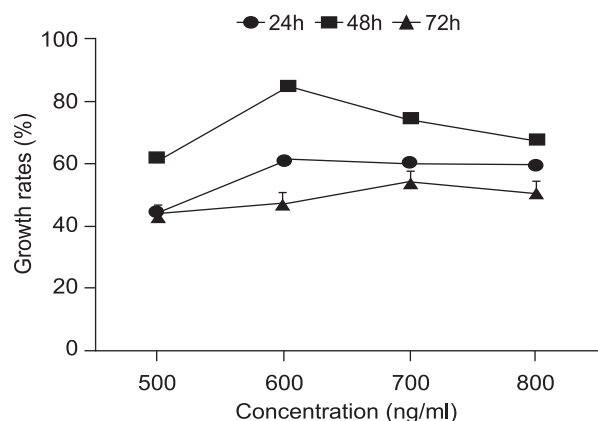


Fig. 3. Growth of OECs treated with 500, 600, 700 and 800 ng/ml ghrelin after 24, 48 and 72 h.

**Ghrelin promotes the growth of OECs via the ERK1/2 pathway:** To identify the signaling pathway through which ghrelin acts on OECs, cells were treated with 600 ng/ml ghrelin for 48 h and the phosphorylation state of the MAPKs ERK1/2, JNK and p38 was examined at 0, 20, 40 and 60 min following treatment. The phosphorylation of ERK1/2

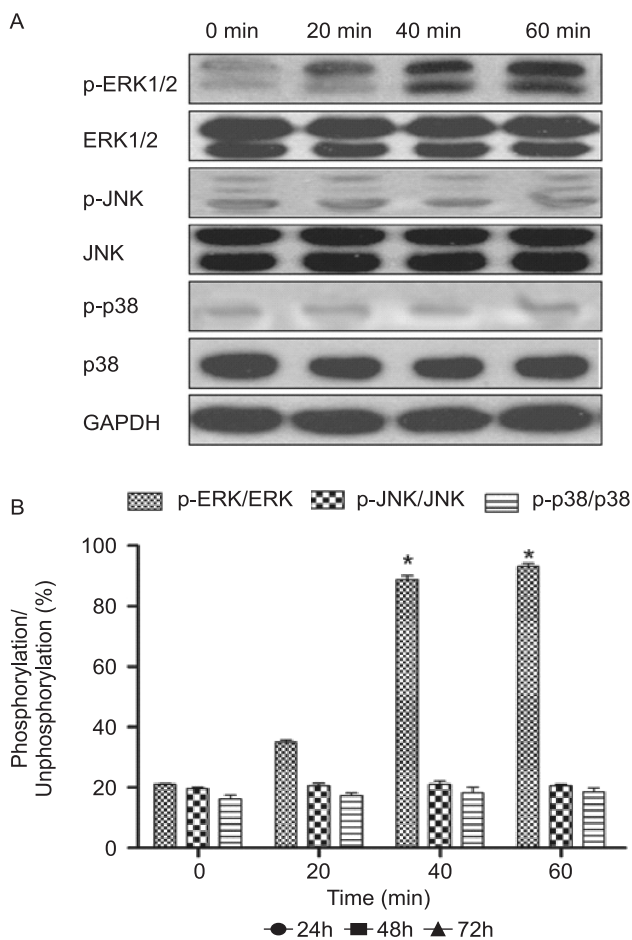


Fig. 4. Effect of 600 ng/ml ghrelin on MAPK activation in OECs at 0, 20, 40 and 60 min. **A**, The expression of ERK1/2, JNKs, and p38 total protein, and phosphorylation of ERK1/2, JNKs, and p38; **B**, densitometric analysis of ERK1/2, JNKs and p38. \*There was a significant difference between 2 groups ( $P < 0.05$ ).

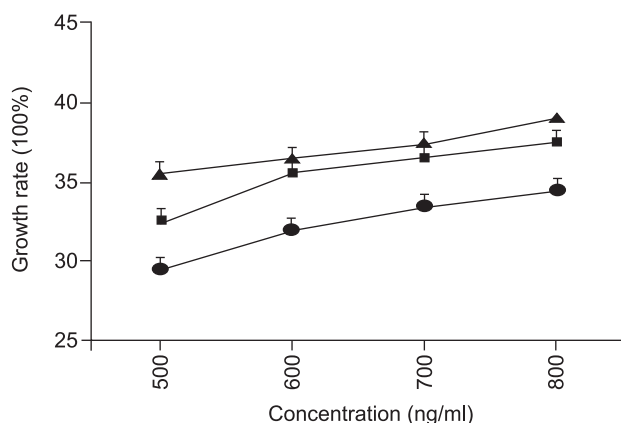


Fig. 5. Growth rates of treated OECs following treatment with 600 ng/ml ghrelin for 24, 48 and 72 h after ERK1/2 inhibited.

increased to a greater extent than that in either JNK or p38 after 40 min (Fig. 4).

To verify that ghrelin promotes the growth of OECs via the ERK1/2 pathway, U0126 (inhibitor specific for phosphorylation ERK1/2) was used to inhibit ERK1/2

activity. Following cells were again treated with ghrelin, and growth was compared to untransfected cells on 24, 48 and 72 h. Inhibition of ERK1/2 eliminated the ability of 600 ng/ml ghrelin to increase the growth of OECs, even after 72 h of treatment (Fig. 5).

The present study investigated the effects of ghrelin on OECs from rodents. Our most important finding is that ghrelin acts as a factor for the proliferation of OECs. This factor can therefore be used in culture techniques aimed at establishing large numbers of ensheathing cells.

The optimal ghrelin treatment to obtain maximum growth of OECs was found to be 600 ng/ml for 48 h. We also demonstrated that GHSR the receptor for ghrelin, is expressed at high levels in OECs, and that the ghrelin induced cellular growth is mediated by and requires, functional GHSR.

To gain further insight into the mechanism through which ghrelin promotes OECs growth, we evaluated the activity of signaling pathways downstream of ghrelin. The MAPKs are members of a superfamily of serine/threonine kinases that includes ERK, JNK and p38 (Silva *et al.* 2014). Our results suggested that ghrelin promotes OECs growth via the ERK signaling pathway, and not through the activation of JNK or p38. Overall, our study suggested that the ghrelin/GHSR signaling pathway accelerated OECs growth mainly through an ERK-dependent pathway. Despite this promising finding, further study is necessary before any clinical application can be considered.

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