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# Ghrelin protects spinal cord motoneurons against chronic glutamate-induced excitotoxicity via ERK1/2 and phosphatidylinositol-3-kinase/Akt/glycogen synthase kinase-3β pathways

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ABSTRACT

Excitotoxic degeneration of spinal cord motoneurons has been proposed as a pathogenic mechanism in amyotrophic lateral sclerosis (ALS). Recently, we have reported that ghrelin, an endogenous ligand for growth hormone secretagogue receptor (GHS-R) 1a, functions as a neuroprotective factor in various animal models of neurodegenerative diseases. In this study, the potential neuroprotective effects of ghrelin against chronic glutamate-induced cell death were studied by exposing organotypic spinal cord cultures (OSCC) to threohydroxyaspartate (THA), as a model of excitotoxic motoneuron degeneration. Ghrelin receptor was expressed on spinal cord motoneurons. Exposure of OSCC to THA for 3 weeks resulted in a significant loss of motoneurons. However, THA-induced loss of motoneurons was significantly reduced by treatment of ghrelin. Exposure of OSCC to the receptor-specific antagonist D-Lys-3-GHRP-6 abolished the protective effect of ghrelin against THA. Treatment of spinal cord cultures with ghrelin caused rapid phosphorylation of extracellular signal-regulated kinase 1/2, Akt, and glycogen synthase kinase-3β (GSK-3β). The effect of ghrelin on motoneuron survival was blocked by the MEK inhibitor PD98059 and the phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002. Taken together, these findings indicate that ghrelin has neuroprotective effects against chronic glutamate toxicity by activating the MAPK and PI3K/Akt signaling pathways and suggest that administration of ghrelin may have the potential therapeutic value for the prevention of motoneuron degeneration in human ALS. Our data also suggest that PI3K/Akt-mediated inactivation of GSK-3β in motoneurons contributes to the protective effect of ghrelin.



## Introduction

Amyotrophic lateral sclerosis (ALS) is a late onset neurodegenerative disease characterized by the selective loss of motoneurons in the spinal cord, brain stem and motor cortex, which causes a progressive muscle weakness and paralysis (Goodall and Morrison, 2006). The mechanism causing motoneuron death in ALS is still mostly unknown; however, excitotoxicity has been implicated in neurodegeneration in this disease (Van Den Bosch et al., 2006). Supporting evidence for this notion is that elevated levels of glutamate in the cerebrospinal fluid (CSF) of ALS patients have been observed, and riluzole, the only drug which proved effective against disease progression in ALS patients, has anti-excitotoxic properties (Bensimon et al., 1994; Miller et al., 2007). It has been reported that glutamate transport in synaptosomes from cerebral cortex and spinal cord of ALS patients is diminished (Rothstein et al., 1992) due to a selective loss of the astroglial glutamate transporter, excitatory amino acid transporter 2/glutamate transporter 1, in the motor cortex and spinal cord (Rothstein et al., 1995). This deficient re-uptake of glutamate results in an excessive concentration of glutamate in the synaptic cleft and the excessive stimulation of glutamate receptors give rise to an increased intracellular concentration of $Na^+$ and $Ca^{2+}$ ions and this can result in excitotoxic neuronal death (Van Den Bosch et al., 2006). Moreover, it was also shown that CSF from ALS patients activated microglial cells and that cytotoxic molecules released from these activated microglia stimulate ionotropic glutamate receptors leading to the death of spinal cord motoneurons (Tikka et al., 2002). Considering that minocycline inhibits the activation of microglial cells and protects motoneurons against excitotoxicity (Tikka et al., 2001, 2002), inhibiting microglial activation which prevents the release of microglia-derived toxic substances could be a plausible therapeutic option.

Ghrelin, an endogenous ligand of growth hormone (GH) secretagogue receptor 1a (GHS-R1a), is a novel GH-releasing acylated peptide that is principally synthesized and released from the stomach (Date et al., 2000). Besides its potent GH-releasing activity, ghrelin has been reported to induce a positive energy balance by stimulating food intake while decreasing fat use through GH-independent mechanisms (Kojima et al., 1999; Peino et al., 2000). Ghrelin also exerts numerous

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peripheral effects including direct effects on exocrine and endocrine pancreatic functions, carbohydrate metabolism, the cardiovascular system, gastric secretion, stomach motility, and sleep (Ghigo et al., 2005; Kojima and Kangawa, 2005; Van der Lely et al., 2004). Recently we have demonstrated that ghrelin exerts neuroprotective effects in different in vivo and in vitro disease models in which excitotoxicity has been involved, including stroke (Chung et al., 2007, 2008; Hwang et al., 2009) and Parkinson's disease (Moon et al., 2009). Moreover, recent reports suggest that the synthetic GHS, GHRP-6 protects neurons from glutamate-induced excitotoxicity (Delgado-Rubin de Celix et al., 2006; Delgado-Rubin et al., 2009) and that ghrelin attenuates kainic acid (KA)-induced hippocampal neuronal cell death in the mouse (Lee et al., 2010a). Given ghrelin receptor GHS-R1a is expressed in the spinal cord (Lee et al., 2010b; Vergnano et al., 2008), these findings suggest that ghrelin could have direct effects on spinal cord function. The phosphatidylinositol-3-kinase (PI3K)/Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways have been implicated in the regulation of cell survival (Datta et al., 1999; Pearson et al., 2001). Our previous studies suggest that these pathways play important roles in the mechanisms of ghrelin-mediated neuroprotection (Chung et al., 2007, 2008). In addition, PI3K/Akt-mediated inactivation of glycogen synthase kinase-3β (GSK-3β) and stabilization of β-catenin also contribute to the neuroprotective activity of ghrelin (Chung et al., 2008). Taken together, these observations prompted us to hypothesize that ghrelin may protect spinal cord motoneurons after glutamate excitotoxicity through the activation of PI3K/Akt and ERK1/2 pathways. Therefore, in the present study, we tested this hypothesis by investigating the role of ghrelin in chronic glutamate-induced excitotoxicity using organotypic spinal cord cultures (OSCCs) exposed to an inhibitor of glutamate transport, threohydroxyaspartate (THA).

## Materials and methods

### *Materials*

Rat ghrelin was obtained from Peptides International (Louisville, KT). D-Lys-3-GHRP-6 was purchased from Bachem (Torrance, CA). Polyclonal rabbit anti-GHS-R1a antibody (FAM-labeled) was obtained from Phoenix Pharmaceuticals (Belmont, CA). Monoclonal antibodies to mouse anti-non-phosphorylated neurofilament H (SMI-32) was purchased from Covance (Princeton, NJ), mouse anti-neuronal nuclei (NeuN) from Millipore (Billerica, MA), and rat anti-glial fibrillary acidic protein (GFAP) was from Invitrogen (Carlsbad, CA). Polyclonal rabbit anti-phospho-antibodies to ERK1/2 (Thr202/Tyr204) and Akt (Ser473), and monoclonal rabbit GSK-3β (Ser9) were purchased from Cell Signaling Technology (Danvers, MA). The PI3K inhibitor, LY294002 and MEK inhibitor, PD98059 were from Tocris (Ellisville, MO). All tissue culture reagents were obtained from Gibco/Invitrogen, and all other reagents were obtained from Sigma unless otherwise indicated.

### *Organotypic rat spinal cord cultures and treatments*

Organotypic spinal cord cultures were prepared from lumbar spinal cords of 8-day-old Sprague–Dawley rat pups (P8), as previously described (Llado et al., 2004). Briefly, pups were decapitated and the lumbar spinal cords were collected under sterile conditions and transferred to sterile Gey's balanced salt solution containing glucose (6.4 mg/ml). They were transversely sectioned at 350 μm by using a McIlwain Tissue Chopper (Mickle Laboratory Engineering, Gomshall, Surrey, UK). Five or six complete slices were transferred to 30-mm-diameter Millipore Millicell®-CM culture plate inserts (0.4 μm, Millipore, Carrightwohill, Co. Cork, Ireland). The inserts were placed in six-well culture plates (35-mm-diameter, Falcon, Becton-Dickinson, Franklin Lakes, NJ, USA), with 1 ml culture medium: 50% (vol/vol) minimal essential medium (MEM) with 25 mM Hepes, 25% (vol/vol) heat-inactivated horse serum and 25% (vol/vol) Hank's balanced salt solution (HBSS) supplemented with D-glucose (25.6 mg/ml, Sigma, St. Louis, MO), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cultures were incubated at 37 °C in a 5% $CO_2$/95% air humidified incubator. Medium was changed twice every week. Under these conditions, cultures can be maintained up to 3 months with steady motoneuron survival and preservation of synaptic morphology. All experiments started at least 7 days after the explant procedure.

Chronic glutamate neurotoxicity was induced by exposure to THA (for 3 weeks), a potent glutamate transporter inhibitor, which is known to produce a dose-dependent sustained elevation of glutamate levels causing degeneration of motoneurons (Rothstein et al., 1993). To determine whether ghrelin inhibits THA-induced motoneuron death in OSCCs, cultures were treated with ghrelin (1, 10, 100 nM) or vehicle at the same time as THA. To examine whether the neuroprotective effect of ghrelin was mediated via its receptor GHS-R1a, OSCCs were pretreated with D-Lys-3-GHRP-6 (100 μM) or vehicle (saline) for 1 h before the treatment with ghrelin. Experiments were also performed by incubating cultures with the following pharmacological inhibitors: 50 μM PD98059 for 1 h or 10 μM LY294002 for 30 min. To investigate the effect of ghrelin on the ERK1/2 and Akt/GSK-3β pathways, 1) cultures were treated with ghrelin (100 nM) for 1, 3, 6, 12, and 24 h; and 2) cultures were preincubated with PD98059 or LY294002, then treated with 100 nM of ghrelin for 6 h and assayed by Western blot analysis described below. All experiments were performed three times, giving essentially identical results.

### *Immunohistochemical detection of GHS-R1a*

Organotypic cultures were fixed with 4% paraformaldehyde (Sigma) in phosphate-buffered saline for 30 min at room temperature (RT). After blocking with 3% normal goat serum (Vector Laboratories, Burlingame, CA) and 1% bovine serum albumin (BSA) (Sigma), the sections were incubated with primary antibodies to SMI-32 (1:8000), NeuN (1:1000), GFAP (1:1000), and GHS-R1a (1:3000) overnight at 4 °C. For immunofluorescence, sections were incubated for 1.5 h at RT with the appropriate secondary antibody, biotinylated anti-goat IgG (1:2000; Vector Laboratories) or cy3-conjugated goat anti-mouse or rat IgG (1:500, Jackson ImmunoResearch, West Grove, PA). Images were acquired by the Carl Zeiss LSM 510 Meta (Oberkochen, Germany) confocal microscope.

### *Motoneuron counts*

Motoneurons in the OSCCs were identified by SMI-32 immunostaining and on the basis of their morphology and size (>25 μm) and their localization in the ventral horn. All motoneurons meeting these criteria were blindly counted in each spinal cord section. A minimum of 30 sections was counted for each experimental condition.

### *Immunohistochemical analysis for subcellular localization of p-Akt, p-ERK1/2 and p-GSK-3β in motoneurons*

For the evaluation of intracellular localization of p-Akt, p-ERK1/2 and p-GSK-3β in spinal cord motoneruons, sections were fixed and probed with antibodies against SMI-32 and p-Akt, p-ERK1/2 or p-GSK-3β, and a secondary antibody conjugated with the fluorescent dye. Fluorescence was captured using a 40X objective lens on a Carl Zeiss LSM 510 Meta confocal microscope (485-nm excitation and 535-nm emission).

*Western blot analysis*

Spinal cord slices 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 $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 10 μg/ml aprotinin. Cell lysates were separated by 12% SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membranes were soaked in blocking buffer (1X Tris-buffered saline, 1% BSA, 1% nonfat dry milk) for 1 h and incubated overnight at 4 °C with the primary antibody. Blots were developed using a peroxidase-conjugated anti-rabbit IgG and a chemiluminescent detection system (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The bands were visualized using a ChemicDoc™ XRS system (Bio-Rad, Hercules, CA) and quantified using Quantity One imaging software (Bio-Rad).

*Statistical analysis*

Data are presented as mean ± SEM. Statistical analysis between 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). $P<0.05$ was considered statistically significant.

## Results

*Ghrelin receptor GHS-R1a is expressed in organotypic spinal cord cultures*

We first examined the expression of ghrelin receptor in our OSCC model. Immunohistochemical analysis revealed GHS-R1a immunoreactivity in these cultures. Double immunolabeling showed that all SMI-32-(+) motoneurons and NeuN-(+) neurons were positive for ghrelin receptor (Fig. 1). In contrast, GHS-R1a did not co-localize with the astrocytes marker GFAP (Fig. 1).

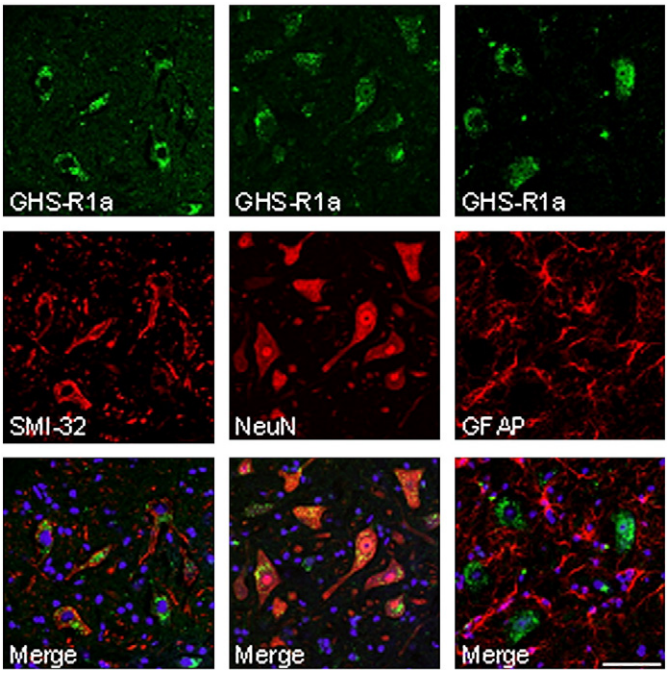


**Fig. 1.** GHS-R1a is expressed in spinal cord motoneurons. Sections were incubated with primary antibodies to GHS-R1a and SMI-32, NeuN, or GFAP. GHS-R1a shows co-localization with SMI-32 and NeuN but not with GFAP. Images were captured using confocal microscopy. Scale bars represent 100 μm.

*Ghrelin protects spinal cord motoneurons against THA-induced chronic glutamate excitotoxicity*

To investigate if ghrelin may act as a survival factor for spinal cord motoneurons, we examined the effect of ghrelin on motoneuron death using OSCC exposed to THA, a glutamate-uptake inhibitor, as an in vitro model of excitotoxic motoneuron degeneration. After 3-week exposure of OSCC to THA (100 μM), 46% of motoneurons were not viable as determined by SMI-32 immunohistochemistry (Fig. 2A). However, THA-induced motoneuron death was significantly reduced in a dose-dependent manner by treatment with ghrelin (1 to 100 nM). The percentage of viable motoneurons compared with vehicle-treated controls decreased to 54% (THA) and was significantly increased to 76% (10 nM) and 95% (100 nM) by treatment with ghrelin. Treatment of OSCC with ghrelin alone had no effect (data not shown). To determine whether the protective effect of ghrelin on motoneurons is mediated by its receptor GHS-R1a, cultures were treated with the receptor specific antagonist D-Lys-3-GHRP-6 (Smith et al., 1993). Exposure of cultures to D-Lys-GHRP-6 (100 μM) completely abolished the protective effect of ghrelin against THA-induced excitotoxic motoneuron degeneration (Fig. 2B). Treatment of OSCC with receptor antagonist alone had no effect on motoneuron degeneration.

*Ghrelin inhibits THA-induced motoneuron death by activating PI3K/Akt and ERK1/2 signaling pathways*

Treatment of organotypic cultures with ghrelin activated Akt in a time- and dose-dependent manner (Figs. 3A and E). Moreover, ghrelin also caused a strong activation of ERK1/2 phosphorylation (Figs. 3B and F). We further determined which signaling pathways are involved in ghrelin-induced Akt and ERK1/2 activation. We found that the PI3K blocker, LY294002 (10 μM), significantly blocked phosphorylation of Akt (Fig. 3G). In contrast, the MEK inhibitor, PD98059 (50 μM), had no effect on ghrelin-stimulated phosphorylation of Akt. Interestingly, ERK1/2 activation by ghrelin was inhibited not only by PD98059, but also by LY294002 (Fig. 3H), suggesting that PI3K is required for activation of the MEK/ERK pathway. To determine if the phosphorylation of these enzymes could be detected in the spinal cord motoneurons in response to ghrelin, double immunohistochemistry using antibodies against SMI-32 and p-Akt or p-ERK1/2 was performed. As shown in Figs. 3C and D, ghrelin increased immunoreactivity for p-Akt or p-ERK1/2 in SMI-32-(+) motoneurons. These data suggest that activation of Akt and ERK1/2 may be involved in the ghrelin-induced protective effect in motoneurons exposed to THA. To test this hypothesis, we examined whether pretreatment of OSCCs with LY294002 (10 μM) or PD98059 (50 μM) impaired the protective effect of ghrelin against THA-induced exciotoxic motoneuron death. We found that both inhibitors significantly blocked the protective effect of ghrelin (Fig. 4). These data suggest that ghrelin inhibits motoneuron death induced by THA via the activation of PI3K/Akt and MAPK/ERK pathways.

*Effect of ghrelin on the regulation of Akt effectors GSK-3β*

Our data demonstrating ghrelin-induced protective effect through the activation of PI3K/Akt pathway suggest that Akt downstream effectors may participate in the neuroprotection of this peptide. One of the ways by which Akt regulates its protective effect is by phosphorylating many effector proteins, such as the cytoplasmic protein kinase GSK-3β. Ghrelin increased phosphorylation of GSK-3β after 3 h treatment and lasted for 24 h (Fig. 5). The inhibition of PI3K/Akt pathway with LY294002 reduced ghrelin-induced GSK-3β phosphorylation to the control levels. To investigate if the phosphorylation of GSK-3β could also be detected specifically in the spinal cord motoneurons after treatment with ghrelin, we performed double immunohistochemistry using antibodies against SMI-32 and p-GSK-3β. Immunoreactivity for phophorylated forms of GSK-3β in

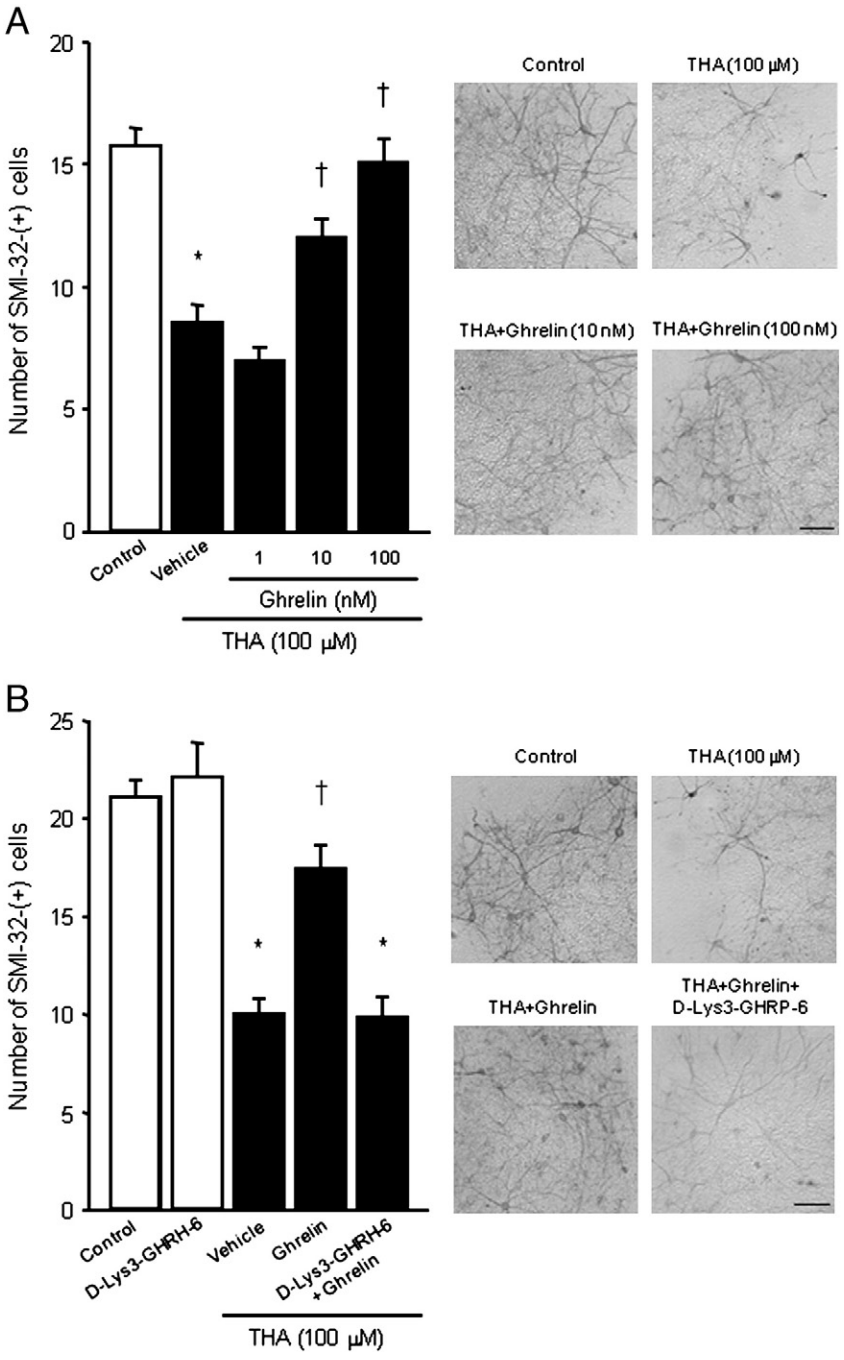


**Fig. 2.** Ghrelin protects spinal cord motoneurons against THA-induced chronic glutamate excitotoxicity. Spinal cord cultures were treated for 3 weeks with 100 μM THA. A, Either vehicle or ghrelin (1, 10, 100 nM) was added to the culture medium at the same time as THA. B, Cultures were preincubated with vehicle or 100 μM D-Lys-3-GHRP-6 for 1 h and then treated with vehicle or 100 nM ghrelin. Motoneurons were identified by SMI-32 immunostaining and on the basis of their morphology, size and location. Representative micrographs of SMI-32-(+) motoneurons in the ventral horn of the spinal cord are shown. Scale bars represent 100 μm. Values are the mean ± SEM of at least 30 sections per treatment. Each experiment was repeated three times. *, $P<0.05$ *vs.* control cultures; †, $P<0.05$ *vs.* THA-insulted, vehicle-treated cultures.

motoneurons was very weak in vehicle-treated group, however, after 6 h treatment with ghrelin, p-GSK-3β immunoreactivity was significantly increased in SMI-32-(+)-motoneurons (Fig. 6A). In contrast, ghrelin-induced phosphorylation of GSK-3β in motoneurons was inhibited by LY294002 (Fig. 6B).

## Discussion

Our recent report (Lee et al., 2010a) demonstrating the role of ghrelin in KA-induced neurotoxicity suggests that ghrelin could be a plausible candidate in the therapy of neurodegenerative diseases, in which excitotoxic cell death is involved. In the present study, we provided evidence that ghrelin protects motoneurons from the chronic glutamate-induced excitotoxicity in organotypic spinal cord cultures through the activation of its receptor GHS-R1a. Our data suggest that ghrelin exerts its protective effect on motoneurons through the activation of the MAPK and PI3K/Akt signaling pathways. We also found that ghrelin-induced stimulation of PI3K/Akt pathways resulted in an inactivation of GSK-3β. Our results suggest that ghrelin can be a potential therapeutic agent in the prevention of motoneuron degeneration in human ALS.

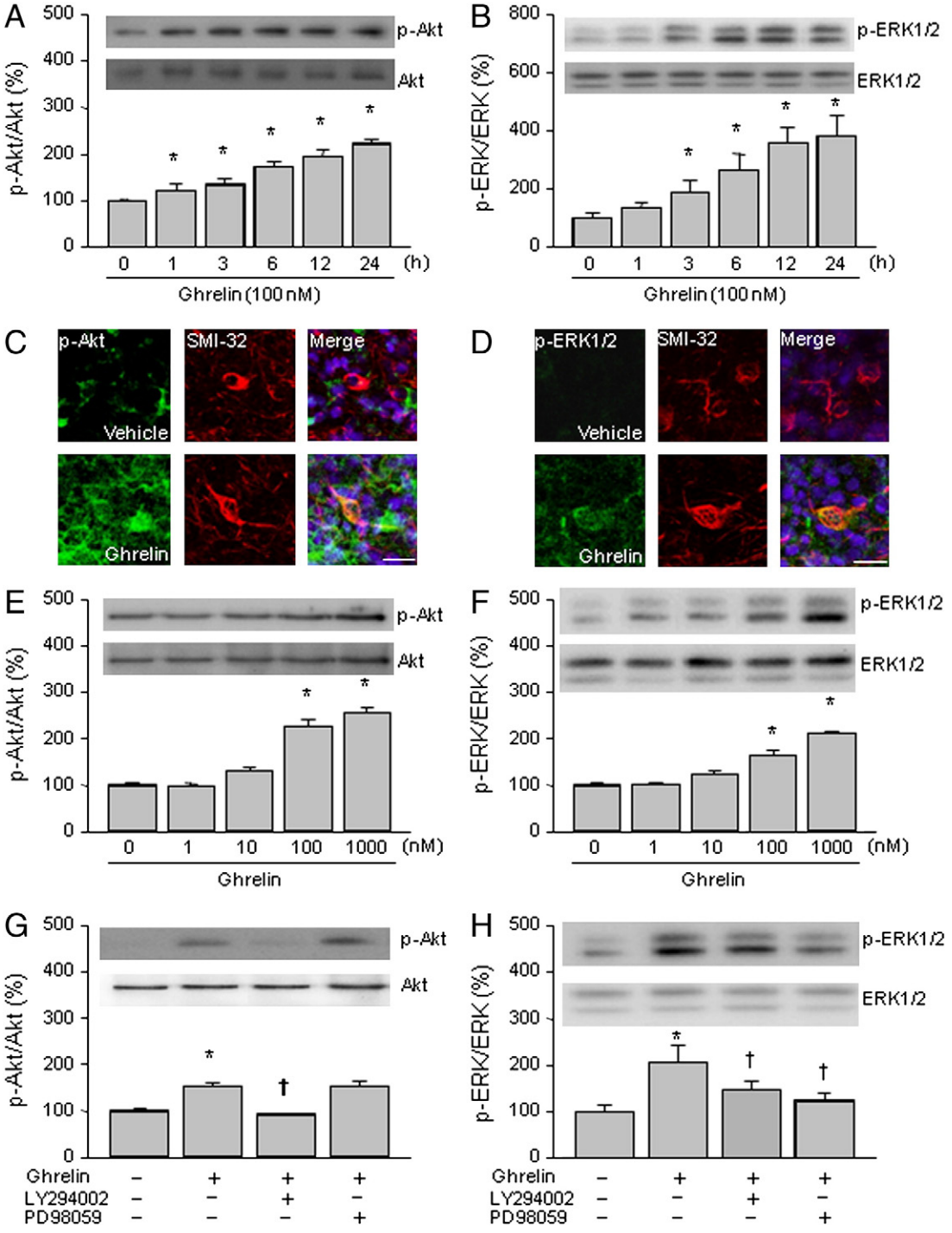


**Fig. 3.** Ghrelin activates Akt and ERK1/2 in organotypic spinal cord cultures. A and B, Time course of ghrelin-induced phosphorylation of Akt (A) and ERK1/2 (B). Cultures were treated with 100 nM ghrelin for 1, 3, 6, 12, and 24 h and assayed by Western blot using specific anti-p-Akt (Ser473) and anti-Akt antibodies (A), and specific anti-p-ERK1/2 (Thr202/Tyr204) and anti-ERK1/2 antibodies (B). C and D, Cultures were treated with 100 nM of ghrelin for 6 h. Slices were fixed and probed with primary antibodies to SMI-32 and p-Akt (C) or p-ERK1/2 (D) to detect localization of phosphorylated Akt or ERK1/2 in motoneurons. Images were captured using confocal microscopy. Scale bars represent 100 μm. E and F, Dose responsiveness of ghrelin-induced phosphorylation of Akt (E) and ERK1/2 (F). Cultures were treated with ghrelin (1 to 1000 nM) for 6 h and assayed by Western blot as above. G and H, PI3K and MAPK pathways mediate ghrelin-induced phosphorylation of Akt (G) and ERK1/2 (H). Cultures were preincubated with 10 μM LY294002 for 30 min or 50 μM PD98059 for 1 h and then treated with 100 nM ghrelin for 6 h and assayed by Western blot as above. The p-Akt and the p-ERK1/2 band intensities were normalized to Akt and ERK1/2 band intensities, respectively, and expressed as % of controls. Values are the mean ± S.E. (n = 4). Each experiment was repeated three times. *, $P<0.05$ vs. untreated cultures; †, $P<0.05$ vs. ghrelin-treated cultures.

Ghrelin protects neurons from various insults such as ischemia, neurotoxicity, and trauma (Chung et al., 2007, 2008; Hwang et al., 2009; Jiang et al., 2008; Lee et al., 2010b; Moon et al., 2009). In the current study, we showed for the first time that ghrelin rescues spinal cord motoneurons from THA-induced chronic excitotoxicity. We have shown the expression of GHS-R1a in spinal cord consistent with our previous report (Lee et al., 2010b). The neuroprotective effect of ghrelin appears to be mediated through the activation of GHS-R1a as we previously reported (Chung et al., 2007, 2008; Hwang et al., 2009; Moon et al., 2009), because the specific antagonist of GHS-R1a

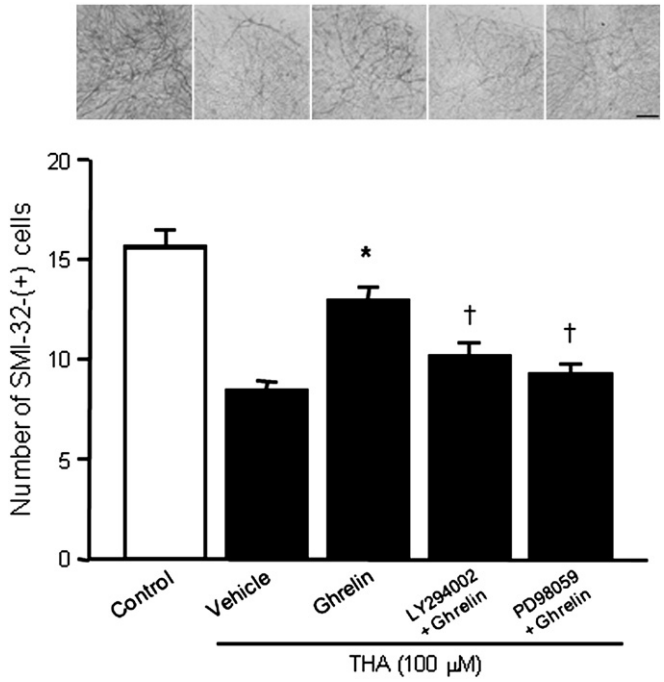


**Fig. 4.** The MAPK and PI3K pathways mediate the protective effects of ghrelin. Organotypic spinal cord cultures were preincubated with 50 μM PD98059 for 1 h or 10 μM LY294002 for 30 min, then treated with 100 M of ghrelin. Spinal cord cultures were exposed to 100 μM THA for 3 weeks. Representative micrographs of SMI-32-(+) motoneurons in the ventral horn of the spinal cord are shown. Scale bars represent 100 μm. Values are the mean ± SEM of at least 30 sections per treatment. Each experiment was repeated three times. *, P<0.05 *vs.* THA-insulted, vehicle-treated cultures; †, *P*<0.05 *vs.* THA-insulted, ghrelin-treated cultures.

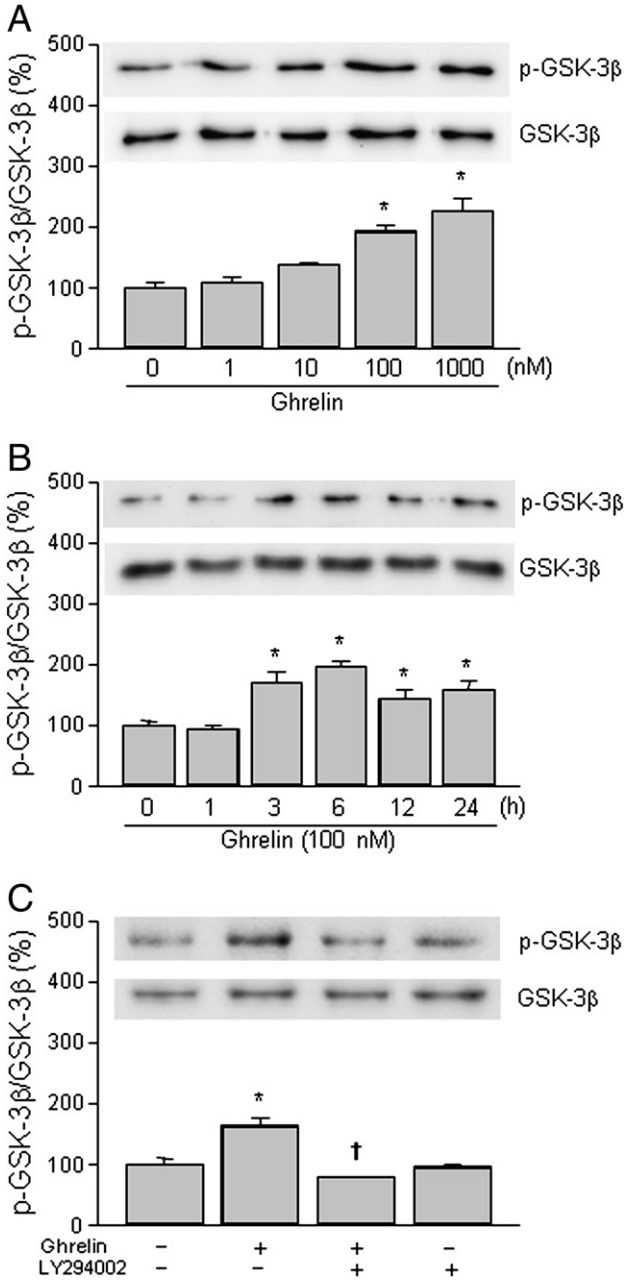


**Fig. 5.** Ghrelin regulates Akt downstream effector GSK-3β in organotypic spinal cord cultures. A, Time-course of ghrelin-induced phosphorylation of GSK-3β. Cultures were treated with 100 nM of ghrelin for 1, 3, 6, 12, and 24 h and assayed by Western blot using specific anti-p-GSK-3β (Ser9) antibodies and anti-GSK-3β antibodies. B, Dose responsiveness of ghrelin-induced phosphorylation of GSK-3β. Cultures were treated with ghrelin (1 to 1000 nM) for 6 h and assayed by Western blot as above. C, PI3K pathways mediate ghrelin-induced phosphorylation of GSK-3β. Cultures were preincubated with 10 μM LY294002 for 30 min and then treated with 100 nM ghrelin for 6 h and assayed by Western blot as above. The p-GSK-3β band intensity was normalized to GSK-3β band intensity, respectively, and expressed as % of controls. Values are the mean ± S.E. (n = 4). Each experiment was repeated three times. *, *P*<0.05 vs. untreated cultures; †, *P*<0.05 vs. ghrelin-treated cultures.

completely blocked the protective effect of ghrelin against THA insult. However, it should be noted that ghrelin could exert its anti-apoptotic effect through binding to a novel, unidentified receptors that is distinct from GHS-R1a (Baldanzi et al., 2002). Considering that ghrelin attenuates ischemia-induced suppression of GHS-R1a mRNA levels (Miao et al., 2007) and hexarelin upregulates the expression of ghrelin receptor (Bresciani et al., 2004; Pang et al., 2004), the regulation of spinal cord GHS-R1a by ghrelin may modulate the local GH or insulin-like growth factor (IGF)-I levels. The spinal cord IGF-I system may be involved in the neuroprotective effect of ghrelin because GHRP-6 increased IGF-I mRNA levels in the brain (Frago et al., 2002) and IGF-I prevented glutamate-induced apoptotic cell death of motoneurons (Vincent et al., 2004). In fact, it has been reported that neurotrophic factors such as ciliary neurotrophic factor, hepatocyte growth factor, and glial cell line-derived neurotrophic factor were upregulated in spinal cord motoneurons of ALS patients (Jiang et al., 2005) and these neurotrophic factors support the survival of spinal motoneurons (Zhang and Huang, 2006). Taken together, our findings provide evidence that ghrelin may act as a survival factor for spinal cord motoneurons and offer a new perspective on the potential role of this peptide in ALS.

Although our data suggest a direct role of ghrelin on spinal cord motoneurons, there is a possibility that the neuroprotective effect of ghrelin may be mediated through the influence on non-neuronal cells, such as microglia and oligodendrocytes. We have recently demonstrated that ghrelin prevents microglial activation induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Moon et al., 2009). Considering that excitotoxic death of motoneurons caused by THA is associated with a neuroinflammatory response characterized by the presence of activated microglia and the concomitant release of pro-inflammatory mediators (Tolosa et al., 2011), the inhibitory effect of ghrelin on microglial activation may play an important role in the neuroprotective effect of ghrelin in chronic glutamate excitotoxicity. In addition, ghrelin may have a direct effect on oligodendrocytes because ghrelin receptors are expressed in these cells (Lee et al., 2010b). Supporting evidence for this assumption is our recent observation that ghrelin inhibits apoptotic cell death of oligodendrocytes after spinal cord injury (Lee et al., 2010b). However, the precise role of ghrelin on microglia and oligodendrocytes remains to be elucidated.

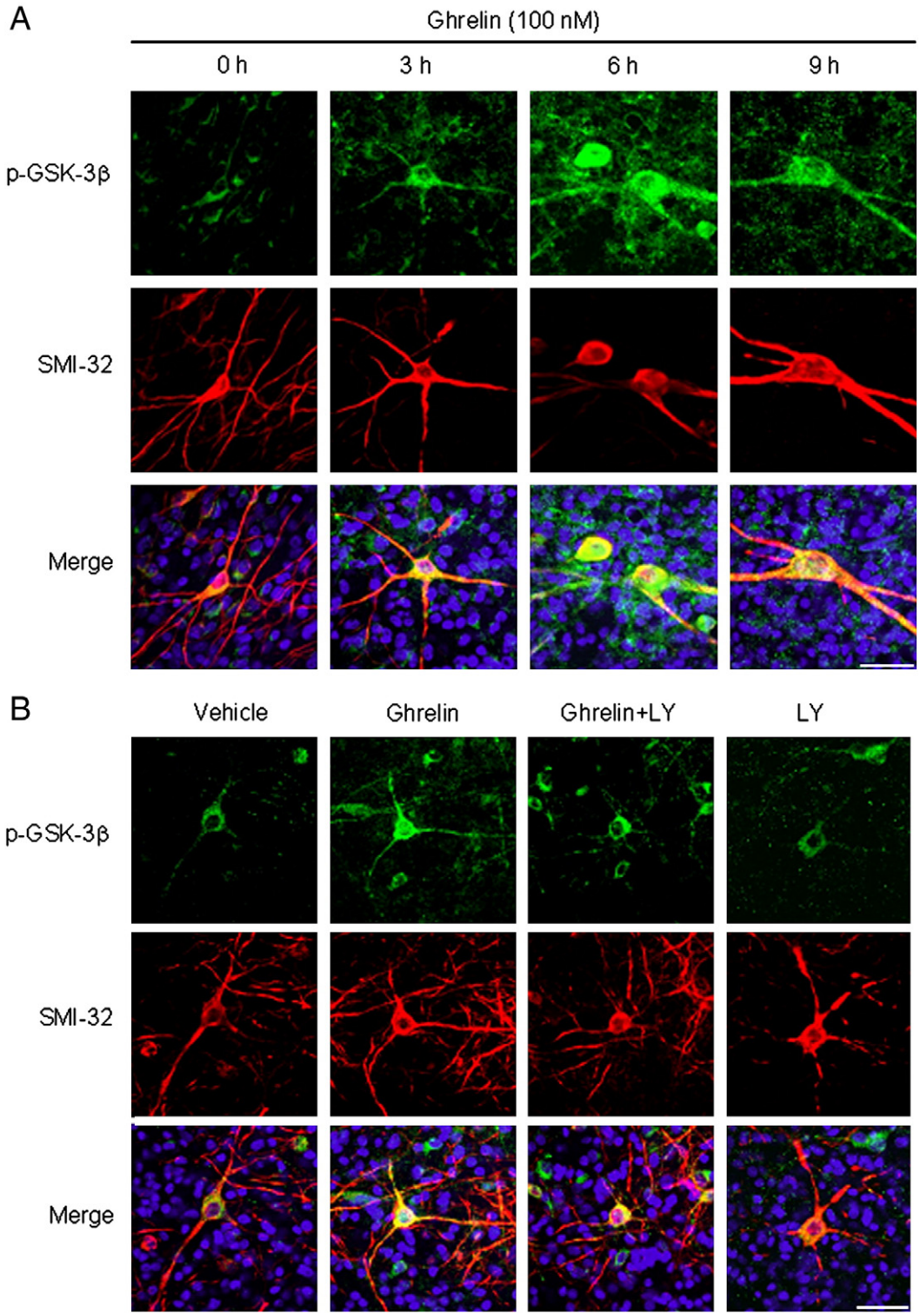


**Fig. 6.** Ghrelin increases phosphorylation of GSK-3β in spinal cord motoneurons. A, Cultures were treated with 100 nM of ghrelin for 3, 6, and 9 h. B, Cultures were preincubated with vehicle or 10 μM LY294002 for 30 min and then treated with 100 nM ghrelin for 6 h. Slices were fixed and probed with primary antibodies to SMI-32 and p-GSK-3β to detect localization of phosphorylated GSK-3β in motoneurons. Images were captured using confocal microscopy. Scale bars represent 100 μm. The results are representative of three independent experiments.

In agreement with our previous report (Chung et al., 2008), we have shown in this study that ghrelin strongly induced the activation of ERK1/2 and Akt in the OSCC. Ghrelin-induced activation of ERK1/2 and Akt also observed in other cell types, such as HIT-T15 cells (Granata et al., 2007) and endothelial cells (Baldanzi et al., 2002). It is well known that ERK1/2 and Akt play important roles in the regulation of cell survival (Datta et al., 1999; Pearson et al., 2001). Selective inhibitors of ERK1/2 and PI3K inhibited ghrelin-induced phosphorylation of ERK1/2 and Akt and the protective effect of ghrelin against chronic glutamate-induced excitotoxicity, indicating that ghrelin suppressed THA-induced cell death in spinal cord motoneuron through the activation of ERK1/2 and PI3K/Akt pathways. Ghrelin

receptor binding activates Gq11, which induces phospholipase C to hydrolyze membrane phosphatidylinositol biphosphate to inositol triphosphate and diacylglycerol. The former mobilizes intracellular $Ca^{2+}$ stores, which together with diacylglycerol, activate protein kinase C (Kojima and Kangawa, 2005). Activation of the GHS-R1a also leads to transactivation of a tyrosine kinase receptor via the β and γ subunits, which activates MAPK via the Ras-Raf-MEK pathway (Nanzer et al., 2004). Moreover, ghrelin exerts its anti-apoptotic effect through the stimulation of cAMP-mediated protein kinase A pathways (Chung et al., 2007; Granata et al., 2007). Collectively, multiple signaling pathways are involved in ghrelin-induced activation of ERK1/2 and Akt, and the protective effect of ghrelin in motoneurons is mediated via the MAPK and PI3K/Akt signaling pathways.

In the current study, we have shown that pretreatment of OSCCs with LY294002 attenuated ERK1/2 phosphorylation in response to ghrelin, in agreement with our previous report demonstrating that chemical inhibition of PI3K activity inhibited ghrelin-induced phosphorylation of ERK1/2 in cortical neuronal cells (Chung et al., 2008). Consistent with our results, previous study demonstrated that wortmannin, the PI3K inhibitor, attenuated ERK1/2 phosphorylation induced by ghrelin in 3T3L1 cells (Kim et al., 2004). Similar findings were also observed in IGF-I-stimulated oligodendrocyte progenitors (Cui and Almazan, 2007). These findings suggest that the PI3K pathway contributes to ghrelin-induced ERK1/2 activation. Indeed, several reports suggested a cross talk between the PI3K and MEK/ERK pathways. Specifically, PI3K activity is essential for the Raf/MEK/ERK cascade activation (Wennstrom and Downward, 1999) and other studies suggest that the PI3K/Akt pathway synergizes with the Raf/MEK/ERK pathway to provide a more robust signal (Rommel et al., 1999; von Gise et al., 2001).

Our results indicate that the PI3K/Akt pathway participates in the ghrelin-mediated neuroprotection against chronic glutamate excitotoxicity. In fact, several studies have also demonstrated that ghrelin-mediated neuronal survival is dependent on PI3K activity (Chung et al., 2008; Xu et al., 2009). Akt is the critical protein activated by PI3K, regulating the balance between cell survival and apoptosis (Song et al., 2005). Although Akt phosphorylation by ghrelin is transient, it may be sufficient to transient signals for survival to downstream targets. This observation is in agreement with Xu et al. (2009), who also showed that a transient phosphorylation of Akt induced by ghrelin is able to rescue hippocampal neurons from pilocarpine-induced toxicity. Thus, the signal transmitted to downstream targets by transient kinase activation is enough to promote long-term survival of neurons. It has been reported that motoneurons of human ALS patients and mutant superoxide dismutase (SOD) 1 mice lose activated Akt and overexpression of constitutively Akt protects against mutant SOD1-dependent cell death (Dewil et al., 2007). These findings suggest that agents upregulating phosphorylated Akt in the spinal cord motoneurons might have clinical relevance for the treatment of ALS.

We previously reported that Akt downstream effector GSK-3β is involved in ghrelin mediated anti-apoptotic effect in cortical neurons during oxygen-glucose deprivation (Chung et al., 2008). Consistent with this report, in the current study, we demonstrate ghrelin phosphorylation of GSK-3β at Ser9 in the spinal cord motoneurons, which was attenuated by pretreatment with LY294002. It also has been shown that ghrelin increases GSK-3β phosphorylation in hippocampal neurons exposed to high glucose (Chen et al., 2010). GSK-3β, a pro-apoptotic protein, is a key downstream target of the PI3K/Akt survival-signaling pathway (Eldar-Finkelman, 2002). It has been demonstrated that GSK-3β activity is required for apoptotic neuronal cell death (Crowder and Freeman, 2000). In addition, KA causes increase in GSK-3β activity in combination with an inactivation of Akt, leading to degeneration of hippocampal neurons (Crespo-Biel et al., 2007). Considering that GSK-3β expression is upregulated in the brain (Yang et al., 2008) and spinal cord (Kihira et al., 2009) of ALS patients and inhibition of GSK-3β by lithium is able to prevent KA-induced excitotoxic motoneuron death (Caldero et al., 2010), our data suggest that PI3K/Akt-mediated inactivation of GSK-3β is at least partly responsible for the neuroprotective effects of ghrelin against THA-induced excitotoxic motoneuron death.

In conclusion, we have demonstrated that ghrelin attenuates spinal cord motoneuron death induced by chronic glutamate excitotoxicity. It is also shown that ghrelin strongly activated ERK1/2 and Akt in motoneurons, and that the protective effect of ghrelin was mediated by the MAPK and PI3K/Akt pathways. Furthermore, we provide evidence that ghrelin-induced Akt signaling is associated with downstream inhibition of GSK-3β in motoneurons. These findings are important because ghrelin can function as a neuroprotective agent and the molecular mechanisms presented in this study may provide another plausible target to prevent progressive motoneuron degeneration in human ALS.

## Disclosure summary

The authors have nothing to disclose.

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