

## Inhibition of Apoptotic Cell Death by Ghrelin Improves Functional Recovery after Spinal Cord Injury

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Spinal cord injury (SCI) induces massive cell death, leading to permanent neurological disability. No satisfactory treatment is currently available. Ghrelin, a gastric hormone, is known to stimulate GH release from the hypothalamus and pituitary gland. Here, we report that ghrelin administration improves functional recovery after SCI in part by inhibiting apoptosis of neurons and oligodendrocytes. Ghrelin was not detected in normal, uninjured spinal cords, but spinal cord neurons and oligodendrocytes expressed the ghrelin receptor. Ghrelin significantly inhibited apoptotic cell death of neurons and oligodendrocytes, release of mitochondrial cytochrome *c*, and activation of caspase-3 after moderate contusion SCI. Ghrelin also significantly increased the level of phosphorylated ERK but decreased the level of phosphorylated p38MAPK. In addition, ghrelin increased the level of ERK-dependent brain-derived neurotrophic factor expression and decreased the level of pronerve growth factor expression. Furthermore, the neuroprotective effects of ghrelin were mediated through the ghrelin receptor. Finally, ghrelin significantly improved functional recovery and reduced the size of the lesion volume and the loss of axons and myelin after injury. These results suggest that ghrelin may represent a potential therapeutic agent after acute SCI in humans. (*Endocrinology* 151: 3815–3826, 2010)

**T**raumatic spinal cord injury (SCI) induces massive cell death, leading to permanent neurological deficits. After SCI, immediate cell death occurs primarily by mechanical insult (1). The primary injury is then followed by a series of secondary events, including glutamate excitotoxicity, ionic dysregulation, ischemia, free radical production, and inflammation, resulting in further cell death (2–4). Apoptosis of neurons and oligodendrocytes after injury is a prominent feature of the secondary degenerative

response, causing progressive degeneration of the spinal cord (5, 6). In particular, a delayed, prolonged death of oligodendrocytes contributes to chronic demyelination and spinal cord dysfunction (7, 8).

Ghrelin, a 28-amino acid gastric hormone, has been identified as an endogenous ligand of the GH secretagogue receptor 1a (GHS-R1a), often referred to as the ghrelin receptor (9). Ghrelin acts on the pituitary and hypothalamus to stimulate GH release, appetite, and adiposity (9–

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For editorial see page 3477

Abbreviations: Akt, Serine-threonine kinase; BBB, Basso-Beattie-Bresnahan; BDNF, brain-derived neurotrophic factor; FG, fluorogold; GFAP, glial fibrillary acidic protein; GHRP, GH-releasing peptide; GHS-R1a, GH secretagogue receptor 1a; GM, gray matter; 5-HT, 5-hydroxytryptamine; JNK, c-Jun N-terminal kinase; MBP, myelin basic protein; MEK, MAPK extracellular kinase-regulated pathway; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NeuN, neuronal nuclei; NF200, 200-kDa neurofilament protein; p, phosphorylated; PD, Parkinson's disease; proNGF, pronerve growth factor; SCI, spinal cord injury; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling; WM, white matter.

13). The endocrine activities of ghrelin are mediated by GHS-R1a, a G protein-coupled receptor expressed mainly in the pituitary, hypothalamus, and hippocampus (14, 15). Although ghrelin is essentially a gastric hormone, it is also expressed in many other tissues, although at lower levels (16). Besides eliciting the release of GH, ghrelin also exerts a broad range of biological actions on the pancreas, carbohydrate metabolism, cardiovascular system, and on gastric secretion and mobility (17–21). Moreover, ghrelin inhibits apoptosis in several types of cells, including cardiocytes, endothelial cells, pancreatic  $\beta$ -cells, and adipocytes (22–24).

Recently, it has been shown that ghrelin inhibits neuronal apoptosis *in vivo* and *in vitro*. An example is the ghrelin inhibition of apoptosis in hypothalamic neurons induced by glucose-oxygen deprivation (25). Ghrelin also prevents cortical neuronal cell death from ischemia (25, 26) and protects dopaminergic neurons from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity (27, 28). Furthermore, GH-releasing peptide (GHRP)-6, an agonist of the ghrelin receptor, attenuates glutamate-induced apoptosis in the hypothalamus and cerebellum (29). Based on these observations, we hypothesized that ghrelin may exert neuroprotective effects after SCI. Here, we showed that systemic administration of ghrelin improved functional recovery after SCI in part by inhibiting apoptotic cell death of neurons and oligodendrocytes. We also found that spinal cord neurons and oligodendrocytes expressed the ghrelin receptor and the neuroprotective effect of ghrelin was mediated through the ghrelin receptor.

## Materials and Methods

### SCI

Adult male Sprague Dawley [Sam: TacN (SD) BR; Samtako, Osan, Korea] rats were subjected to moderate contusion injury (10 g  $\times$  25 mm) as described previously (8). Surgical interventions and postoperative animal care were performed in accordance with the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care Committee of the Kyung Hee University.

### Drug administration

Acylated ghrelin (Peptides International, Louisville, KY) and an antagonist to the ghrelin receptor, [D-Lys3]-GHRP-6 (Bachem, Torrance, CA), were dissolved in sterile PBS and injected ip into animals. Rats were given ghrelin (40, 80, or 160  $\mu$ g/kg) immediately after SCI and then received the same dose of ghrelin every 6 h for 1 d. Preliminary studies showed that a dose of 80  $\mu$ g/kg of ghrelin was an optimal dose for neuroprotection after injury. Thus, we used 80  $\mu$ g/kg of ghrelin throughout experiments. For the sham-operated controls, the animals underwent a thoracic 9–10 laminectomy without contusion injury and

received no pharmacological treatment. [D-Lys3]-GHRP-6 (1, 3, and 5 mg/kg, ip) (30) was given ip immediately before ghrelin treatment after injury. To determine the therapeutic window of ghrelin, ghrelin (80  $\mu$ g/kg) was administered at 2, 6, 12, or 24 h after SCI and then continued every 6 h at 80  $\mu$ g/kg for an additional day. Control groups received equivolumetric ip injections of PBS at the corresponding time points. Intraspinal injection of PD98059 (Calbiochem, La Jolla, CA), an inhibitor of MAPK extracellular kinase-regulated pathway (MEK)/ERK, was administered as previously described (31). In brief, 2  $\mu$ l of PD98059 (30  $\mu$ mol) dissolved in 50% dimethylsulfoxide were injected into the epicenter of the lesion at 1 h after SCI. Control groups received injections of equal volumes of 50% dimethylsulfoxide at the corresponding time points.

### Tissue preparation

At specific time points after SCI, animals were anesthetized with chloral hydrate and perfused via cardiac puncture. A 20-mm section of the spinal cord, centered at the lesion site, was dissected out, postfixed by immersion in the same fixative overnight, and placed in 30% sucrose in 0.1 M PBS, pH 7.4. The segment was embedded in optimal cutting temperature for frozen sections, and longitudinal or transverse sections were then cut at 10 or 20  $\mu$ m on a cryostat (CM1850; Wetzlar, Leica, Solms, Germany).

### Immunohistochemistry

Frozen sections were processed for immunohistochemistry with antibodies against GHS-R1a (1:300; Phoenix Pharmaceuticals, Inc., Burlingame, CA), brain-derived neurotrophic factor (BDNF) (1:1000; Millipore, Billerica, MA), and myelin basic protein (MBP) (1:1000; Millipore) as previously described (8). Some sections stained for GHS-R1a or BDNF were double labeled using antibodies specific for identifying neurons [neuronal nuclei (NeuN), 1:100; Millipore], astrocytes [glial fibrillary acidic protein (GFAP); 1:1000; Millipore], microglia (OX-42, 1:100; Millipore), or oligodendrocytes (CC1, 1:100; Oncogene, Cambridge, MA). For double-labeling, fluorescein isothiocyanate or cy3-conjugated secondary antibodies were used (Jackson ImmunoResearch, West Grove, PA). Also, nuclei were labeled with 4',6-diamidino-2-phenylindole according to the protocol of the manufacturer (Molecular Probes, Eugene, OR). Immunostaining control studies were performed by omission of the primary antibodies, by replacement primary antibodies with non-immune, control antibody, and by preabsorption with an excess (10  $\mu$ g/ml) of the respective antigens. Serial sections were also stained for histological analysis with Cresyl violet acetate.

### Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL)

One and 5 d after injury, serial spinal cord sections (20- $\mu$ m thickness) were collected, and every 100- $\mu$ m section was processed for TUNEL and then for immunocytochemistry using specific cell type markers: anti-NeuN (1:1000) and anti-CC1 (1:100). TUNEL was performed according to the manufacturer's instruction (Oncor, Gaithersburg, MD). The negative control sections were treated similarly but incubated in the absence of terminal deoxynucleotidyl transferase enzyme, deoxyuridine triphosphate-digoxigenin, or antidigoxigenin antibody, and the positive control sections were incubated in deoxyribonuclease 1.

Only double-labeled cells were considered and counted as TUNEL-positive neurons (1 d) in the gray matter (GM) or oligodendrocytes (5 d) in the white matter (WM). In addition, only those cells showing morphological features of nuclear condensation and/or compartmentalization only in the GM and the WM were counted as TUNEL-positive. To avoid any bias in the results, cell counts were done blind by the persons who did not know the treatment history of the animals; no results were revealed to the counters until the completion of data collection for the entire study.

### RNA isolation and RT-PCR

For details on RNA isolation and RT-PCR, see Supplemental Methods published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>.

### Western blot analysis

Segments of spinal cord (1 cm) were isolated using the lesion site as the epicenter, and the tissue homogenates were prepared as previously described (8, 32). Protein extraction of both the mitochondrial and the cytosolic fractions was performed as previously described (33). Total (50  $\mu$ g), cytosolic (25  $\mu$ g), and mitochondrial (10  $\mu$ g) protein was separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore) by electrophoresis. The membranes were then incubated with antibodies against cytochrome *c* (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), ghrelin (1:500; Santa Cruz Biotechnology), GHS-R1a (1:500; Santa Cruz Biotechnology), Bcl-2 (1:500; Santa Cruz Biotechnology), Bax (1:500; Santa Cruz Biotechnology), BDNF (1:1000; Millipore), MBP (1:1000; Millipore), cleaved caspase-3 (1:1000; Cell Signaling Technology, Danvers, MA), ERK (1:5000; Cell Signaling Technology), phosphorylated (p)-ERK (1:5000; Cell Signaling Technology), p38MAPK (1:1000; Cell Signaling Technology), p-p38MAPK (1:1000; Cell Signaling Technology), c-Jun N-terminal kinase (JNK) (1:3000; Cell Signaling Technology), p-JNK (1:3000; Cell Signaling Technology), serine-threonine kinase (Akt) (1:1000; Cell Signaling Technology), p-Akt (1:1000; Cell Signaling Technology), and pronerve growth factor (proNGF) (1:1000; Alomone Labs, Jerusalem, Israel).  $\beta$ -Tubulin (1:10,000; Sigma, St. Louis, MO) and cytochrome *c* oxidase IV (1:5000; Invitrogen) were used as an internal control. Densitometric quantification of all of the bands on Western blottings was done using AlphaImager software (Alpha Innotech Corp., Santa Clara, CA). Background in films was subtracted from the OD measurements.

### DNA laddering

Segments of spinal cord (1 cm) were isolated using the lesion site as the epicenter at 1 or 5 d after SCI, and DNA was separated by electrophoresis as previously described (32). For details, see Supplemental Methods.

### Behavioral tests

Behavioral analyses were performed according to previously described (34–37).

### Statistical analysis

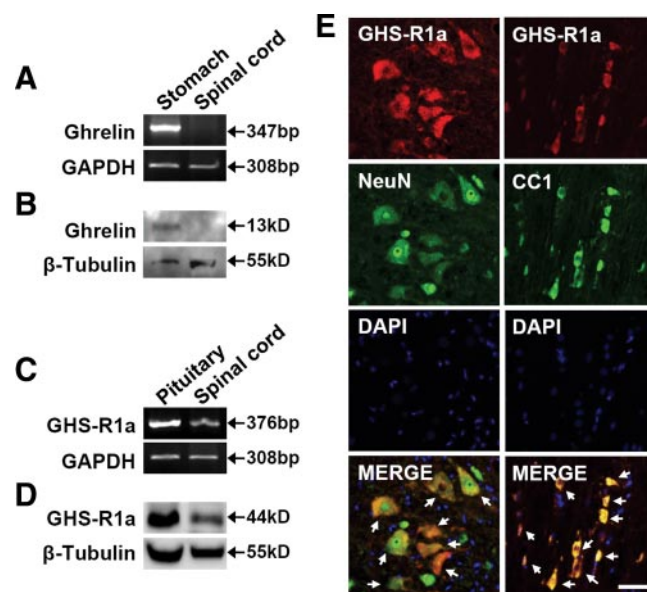
Data presented as the mean  $\pm$  SD values. Comparisons between vehicle- and ghrelin-treated groups were made by unpaired Student's *t* test. Multiple comparisons between groups were performed one-way ANOVA. Behavioral scores from Bas-

so-Beattie-Bresnahan (BBB) analysis and inclined plane tests were analyzed by repeated measures ANOVA (time *vs.* treatment). Tukey's multiple comparison was used as *post hoc* analysis. Statistical significance was accepted with  $P < 0.05$ . All statistical analyses were performed by using SPSS 15.0 (SPSS Science, Chicago, IL).

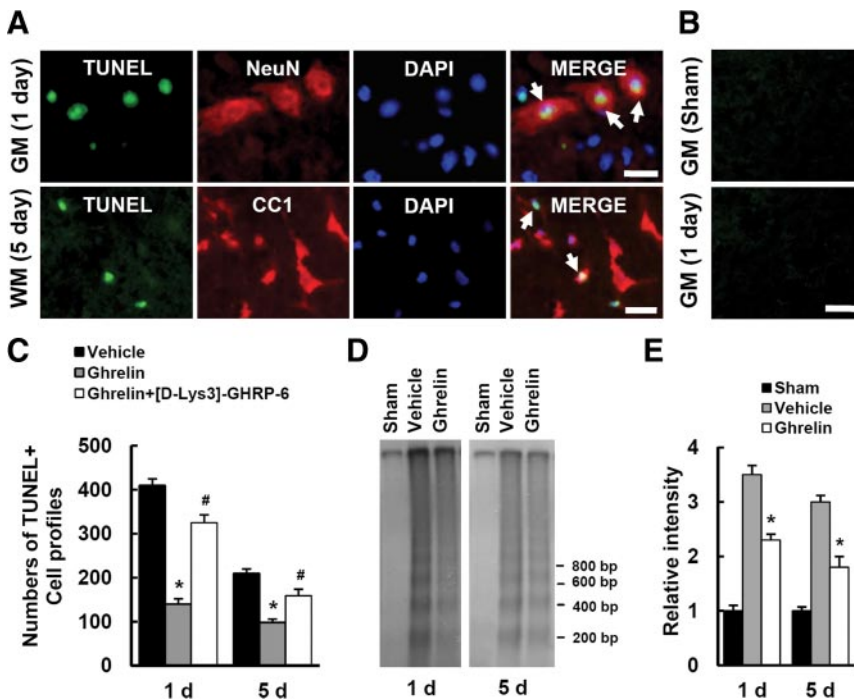
## Results

### Spinal cord neurons and oligodendrocytes express ghrelin receptors

Ghrelin is essentially a GH-releasing, gastric hormone (9); its endocrine activities are mediated by GHS-R1a, a G protein-coupled receptor expressed mainly in the pituitary and hypothalamus (14, 15). We first investigated whether the rat spinal cord expresses ghrelin and GHS-R1a. RT-PCR and Western blot analyses revealed that GHS-R1a mRNA and protein were expressed in normal spinal cords, whereas ghrelin mRNA and protein expression were not detected (Fig. 1, A–D). Next, to ascertain the cell types expressing GHS-R1a, we performed immunostaining using an antibody specific for GHS-R1a accompanied by such cell-type specific markers as NeuN for neurons, CC1 for oligodendrocytes, GFAP for astrocytes, and OX-42 for microglia. Double-labeling revealed that all neurons in the GM and oligodendrocytes in the WM were positive for



**FIG. 1.** Expression of ghrelin and ghrelin receptor, GHS-R1a in normal spinal cord. Total mRNA and protein from normal spinal cord tissue, stomach (as a positive control for ghrelin), and pituitary (as a positive control for GHS-R1a) were prepared, and RT-PCR and Western blot analysis were performed as described in *Materials and Methods* ( $n = 3$ ). Ghrelin mRNA (A) and protein (B) were not expressed in the normal spinal cord. However, GHS-R1a mRNA (C) and protein (D) were detected in the spinal cord. E, Representative fluorescence microscopic photographs show that NeuN-positive neurons (arrows in left panel) and CC1-positive oligodendrocytes (arrows in right panel) are positive for GHS-R1a. DAPI, 4',6-Diamidino-2-phenylindole. Scale bar, 30  $\mu$ m.



**FIG. 2.** Effect of ghrelin on apoptotic cell death of neurons and oligodendrocytes after SCI. Vehicle-, ghrelin- (80  $\mu$ g/kg), and ghrelin receptor antagonist-, [D-Lys3]-GHRP-6 (3 mg/kg), treated spinal cord sections at 1 d or 5 d after SCI were processed for TUNEL staining as described in *Materials and Methods* ( $n = 5$ ). *A*, *Upper panels*, TUNEL-positive neurons (NeuN) in the ventral horn area at 1 d after injury. Representative images were from the sections selected 1 mm rostral to the lesion epicenter. *A*, *Bottom panels*, TUNEL-positive oligodendrocytes (CC1) in the WM (ventral funiculus area) at 5 d after SCI. Representative images were from the sections selected 5 mm rostral to the lesion epicenter. DAPI, 4',6-Diamidino-2-phenylindole. *Scale bars*, 20  $\mu$ m. *B*, Any positive signal was not observed in both sham (*upper panel*) and terminal deoxynucleotidyl transferase enzyme absent negative control (*bottom panel*). *Scale bar*, 20  $\mu$ m. *C*, Quantitative analysis of numbers of TUNEL-positive cell profiles shows that ghrelin treatment significantly decreased the numbers of TUNEL-positive neurons at 1 d and oligodendrocytes at 5 d after injury. The ghrelin receptor antagonist treatment significantly alleviated the antiapoptotic effect of ghrelin after injury. For quantification, serial transverse sections (20- $\mu$ m thickness) were collected every 100- $\mu$ m section from 2 mm rostral to 2 mm caudal to the lesion epicenter (total 40 sections for neurons) or 5 mm rostral to 5 mm caudal to the lesion epicenter (total 100 sections for oligodendrocytes). The double (NeuN + TUNEL) positive neurons in the ventral horn area and oligodendrocytes in the WM from each section were counted. Data represent mean  $\pm$  SD obtained from five separate experiments. \*,  $P < 0.01$  compared with vehicle; #,  $P < 0.05$  compared with ghrelin. *D*, DNA was isolated from spinal cord tissues at 1 d or 5 d after SCI and labeled with [ $\alpha$ - $^{32}$ P]-dCTP ( $n = 3$ ). The extent of DNA laddering was increased after injury compared with sham control. The gels are representative results from three separate experiments. *E*, Quantitative analysis of DNA laddering gels shows that ghrelin treatment significantly decreased the extent of laddering compared with vehicle after injury. Values are means  $\pm$  SD of three separate experiments. \*,  $P < 0.01$ .

GHS-R1a (Fig. 1E), whereas astrocytes and microglia were negative (data not shown).

### Ghrelin inhibits apoptotic cell death of neurons and oligodendrocytes after SCI

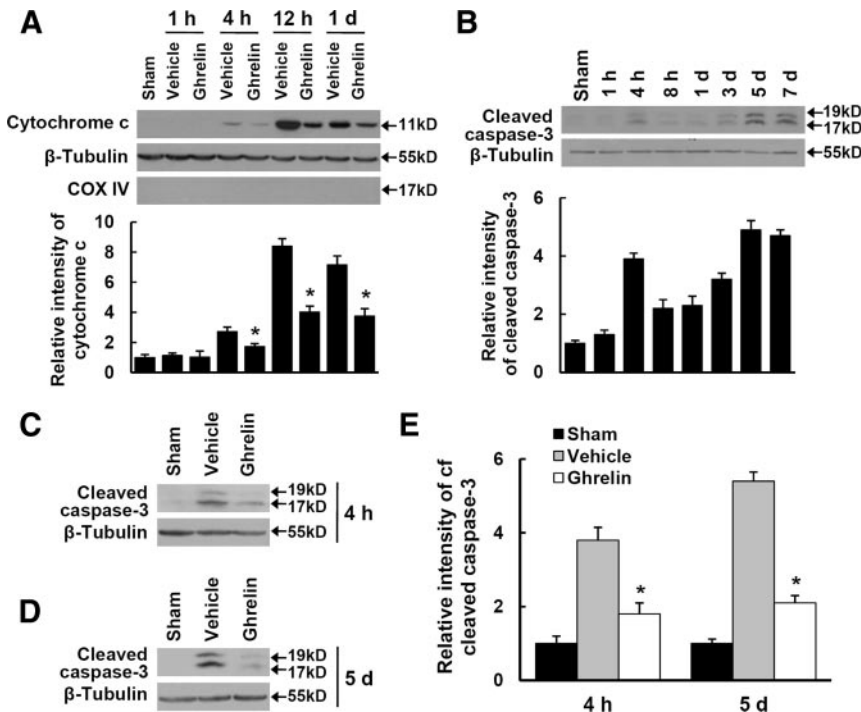
Because ghrelin is known to inhibit apoptosis in several cell types, including neurons (14, 22, 23, 25, 38), we hypothesized that ghrelin would inhibit apoptotic cell death after SCI. Many TUNEL-positive cells were seen mostly within the lesion area in the GM at 1 d and observed in the WM at 5 d mostly outside of the lesion area extending the

entire length of the section (20 mm). However, TUNEL-positive cells were not observed in the sham-operated or negative control (Fig. 2B). Double-labeling confirmed that a number of TUNEL-positive neurons at 1 d and oligodendrocytes at 5 d after injury were observed as previously reported (Fig. 2A) (8, 32). Ghrelin treatment after injury significantly decreased the numbers of cell profiles of both TUNEL-positive neurons at 1 d and oligodendrocytes at 5 d when compared with the vehicle-treated control (Fig. 2C). It should be noted that TUNEL can detect not only apoptotic DNA but also the random fragmentation of DNA by multiple endonucleases occurring in cellular necrosis. However, the necrotic cell death after SCI is prominent at the lesion epicenter (39, 40), and the apoptosis of neurons and oligodendrocytes is a prominent feature of the secondary degeneration after SCI (6, 37, 41, 42). However, we observed that most of TUNEL+/NeuN+ and TUNEL+/CC1+ cells existed in the perilesional area, not lesion epicenter (data not shown). Therefore, we consider most of TUNEL-positive neuron and oligodendrocytes counted were underwent apoptosis. DNA gel electrophoresis also revealed that posttreatment with ghrelin significantly reduced DNA laddering at 1 and 5 d compared with the vehicle-treated control (Fig. 2, D and E).

### Ghrelin inhibits cytochrome *c* release and caspase-3 activation after SCI

Mitochondrial cytochrome *c* release and caspase-3 activation after SCI occur at an early stage of apoptotic cell death (32, 43–46). Because we found

the neuroprotective effect of ghrelin after injury (see Fig. 2C), we anticipated that ghrelin treatment would inhibit cytochrome *c* release and caspase-3 activation. Western blot analysis revealed that cytochrome *c* release into cytoplasm after injury was increased from 4 h and peaked at 12 h (Fig. 3A). Posttreatment with ghrelin significantly decreased mitochondrial cytochrome *c* release when compared with the vehicle-treated control (Fig. 3A). Western blot analysis also revealed that the level of cleaved (activated) forms of caspase-3 was increased and peaked at 4 h



**FIG. 3.** Ghrelin inhibits cytochrome *c* release and caspase activation after SCI. Spinal cord samples at 1 h, 4 h, 12 h, 1 d, 3 d, 5 d, and 7 d after injury were processed for Western blot analysis as described in *Materials and Methods* ( $n = 3$ ). A, Western blot analysis of cytochrome *c* with cytoplasmic fractions after injury. Quantitative analysis of Western blottings shows that ghrelin treatment significantly decreased the level of cytochrome *c* release into cytoplasm compared with vehicle treatment after injury. \*,  $P < 0.05$ . B, Western blot analysis of activated caspase-3 after injury. Quantitative analysis of Western blottings shows that the level of active caspase-3 was increased at 4 h and 5 d after SCI. Western blottings of activated caspase-3 at 4 h (C) and 5 d (D) after ghrelin treatment. E, Quantitative analysis of Western blottings shows that ghrelin treatment significantly decreased the level of activated caspase-3 at 4 h and 5 d compared with vehicle treatment after injury. Values are mean  $\pm$  SD of three separate experiments. \*,  $P < 0.05$ .

and 5 d after injury (Fig. 3B). Furthermore, ghrelin treatment also significantly decreased the level of caspase-3 activation when compared with the vehicle-treated control (Fig. 3, C–E). These results indicate that ghrelin treatment inhibits cytochrome *c* release and caspase-3 activation after injury as previously reported (25, 27).

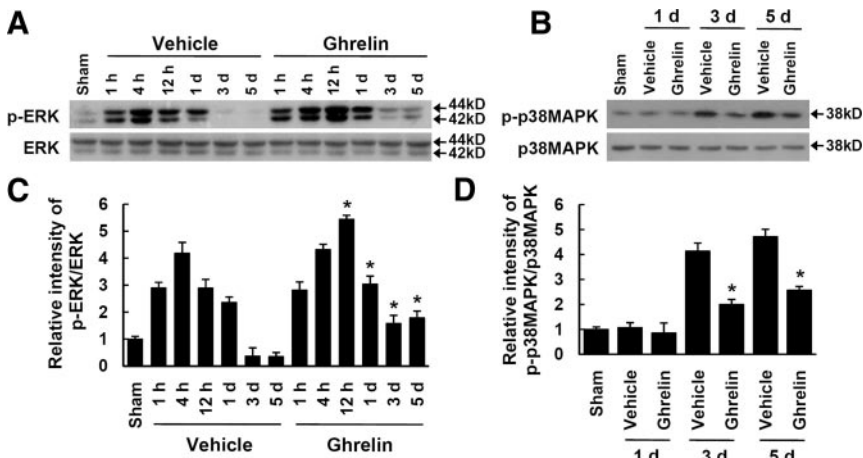
### Ghrelin activates ERK and inhibits p38MAPK after SCI

Among the members of the MAPK family, ERK, p38MAPK, and JNK are known to be associated with cell death or survival after SCI (8, 31, 47). As we demonstrated that ghrelin treatment after SCI inhibited apoptotic cell death of neurons and oligodendrocytes (see Fig. 2), we postulated that ghrelin would modulate MAPK signaling as well. Western blot analysis revealed that the level of p-ERK was markedly increased and peaked at 4 h after injury, but the level of total ERK was not changed as previously reported elsewhere (Fig. 4A) (14, 31). Similarly, the level of p-p38MAPK was also increased and peaked at 5 d after injury, but the level of total p38MAPK was not

changed as previously reported elsewhere (Fig. 4B) (8). Ghrelin treatment after injury significantly increased the level of p-ERK and decreased the level of p-p38MAPK when compared with the vehicle-treated control (Fig. 4, A–D). However, neither injury nor ghrelin treatment after injury changed the level of p-JNK (Supplemental Fig. 1A). Furthermore, the level of p-Akt, which is known to be associated with cell survival after SCI (31), was not changed by ghrelin treatment compared with the vehicle-treated control (Supplemental Fig. 1B). These results suggest that the antiapoptotic effect of ghrelin may be mediated in part through ERK and p38MAPK signaling as previously reported elsewhere (24, 25, 29).

### Ghrelin increases ERK-dependent BDNF expression and decreases p38MAPK-dependent proNGF expression after SCI

Our previous study demonstrated that the activation of ERK is involved in neuronal survival after SCI (31). However, the mechanisms underlying ERK-mediated neuronal survival after injury are largely unknown. A recent report showed that the activation of MEK/ERK pathway increases BDNF expression, which is involved in survival of NM9D dopaminergic cells from 6-hydroxydopamine toxicity (48). It has also been known that the expression of BDNF is increased after SCI (49, 50). Furthermore, the administration of BDNF promotes neuronal and oligodendroglial survival (51, 52) and improves the recovery of locomotor functions after SCI (53, 54). Therefore, we hypothesized that ERK-dependent BDNF expression after SCI may mediate the antiapoptotic effect of ghrelin. We first examined the expression profile of BDNF after injury. RT-PCR analysis revealed that after SCI, BDNF mRNA was increased and peaked at 1 d (Fig. 5A) as previously reported (49, 50). Western blot analysis, using an antibody against an active form of BDNF (14 kDa), also revealed that BDNF protein was increased and peaked at 1 d (Fig. 5B). Furthermore, double-labeling revealed that after injury, all neurons and oligodendrocytes were positive for BDNF, whereas astrocytes and microglia were negative for BDNF (Fig. 5C). Next, we examined the effect of ghrelin on BDNF expression after SCI. Ghrelin treatment after injury significantly



**FIG. 4.** Ghrelin increases ERK activation and inhibits p38MAPK activation after SCI. Spinal cord tissue extracts were prepared as described in *Materials and Methods* ( $n = 3$ ). The gels presented are representative of results from three separate experiments. A, Western blot analysis of p-ERK. B, Western blot analysis of p-p38MAPK. C, Quantitative analysis of Western blottings shows that ghrelin treatment significantly increased the level of p-ERK compared with vehicle after injury. Values are means  $\pm$  SD of three separate experiments. \*,  $P < 0.01$ . D, Quantitative analysis of Western blottings shows that ghrelin treatment significantly decreased the level of p-p38MAPK compared with vehicle after injury. Values are means  $\pm$  SD of three separate experiments. \*,  $P < 0.01$ .

increased the expression of both BDNF mRNA and protein at 1 d (Fig. 5, D and E). By contrast, ghrelin treatment did not change the level of BDNF in uninjured spinal cord (data not shown). Moreover, treatment with PD98059 (30  $\mu$ mol), an inhibitor of MEK/ERK, significantly decreased the levels of both p-ERK and BDNF expression at 1 d after injury (Fig. 5F). These results suggest that the antiapoptotic effects of ghrelin may be mediated in part by increasing ERK-dependent BDNF expression after SCI.

Our previous report showed that proNGF production via activation of p38MAPK in microglia after SCI is involved in oligodendrocyte cell death (8). Because we showed that ghrelin inhibited p38MAPK activation after injury (see Fig. 4, C and D), we hypothesized that ghrelin would inhibit proNGF expression after SCI. Western blot analysis revealed that proNGF expression was increased at 5 d after injury as reported previously (8). Ghrelin treatment significantly decreased the level of proNGF expression when compared with vehicle-treated control (Fig. 5G). These results suggest that ghrelin may inhibit apoptotic cell death of oligodendrocytes in part by decreasing p38MAPK-dependent proNGF expression after SCI.

#### Ghrelin increases *IGF-I* expression after SCI

It has been shown that ghrelin increases the level of IGF-I in various brain regions (55), and IGF-I increases neuronal survival and improves functional recovery after SCI (56–58). To determine whether ghrelin modulates the expression of *IGF-I* after SCI, RT-PCR for *IGF-I* was performed. As shown in Supplemental Fig. 2, the level of *IGF-I* mRNA expression was increased by ghrelin treat-

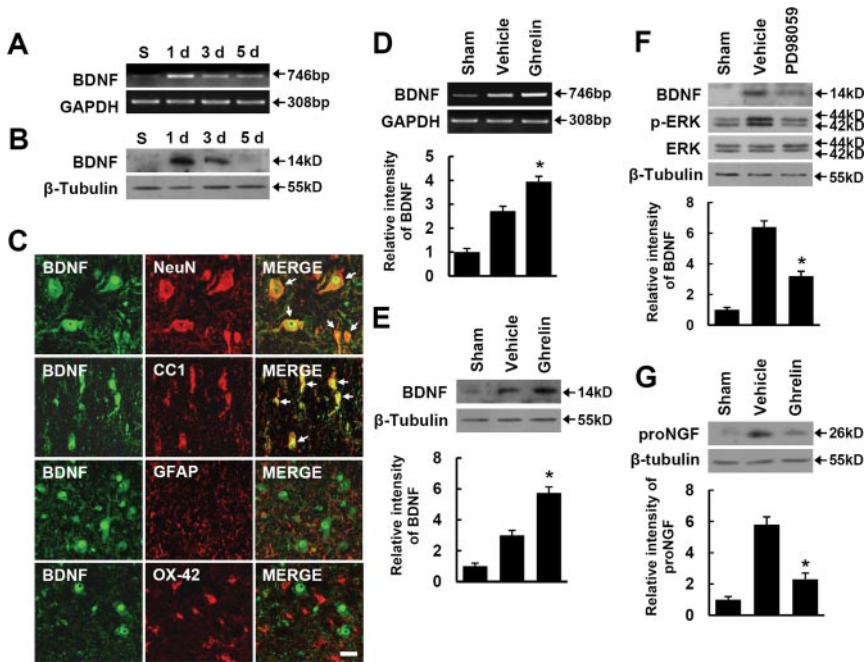
ment after SCI. These results suggest that the neuroprotective effect of ghrelin may be mediated in part by increasing *IGF-I* expression after SCI.

#### Ghrelin receptor mediates the antiapoptotic effect of ghrelin after SCI

Because spinal cord neurons and oligodendrocytes expressed GHS-R1a (see Fig. 1), we hypothesized that the antiapoptotic effect of ghrelin after injury may be mediated through the ghrelin receptor. We administered the specific ghrelin receptor antagonist, [D-Lys3]-GHRP-6 (1, 3, and 5 mg/kg, ip) (30), immediately before ghrelin treatment after SCI. As shown in Fig. 6, A and B, [D-Lys3]-GHRP-6 (3 or 5 mg/kg) significantly alleviated the inhibitory effect of ghrelin on caspase-3 activation at 4 h and 5 d after injury. Also, [D-Lys3]-GHRP-6 (3 mg/kg) significantly mitigated the stimulatory effect of ghrelin on ERK activation at 4 h and the inhibitory effect of p38MAPK activation at 5 d after injury (Fig. 6, C and D). Furthermore, [D-Lys3]-GHRP-6 (3 mg/kg) treatment significantly reduced the antiapoptotic effect of ghrelin on neuronal and oligodendroglial cell death (Fig. 2C). These results indicate that the antiapoptotic effect of ghrelin might be mediated through the ghrelin receptor.

#### Ghrelin improves functional recovery after SCI

Because ghrelin inhibited neuronal and oligodendroglial apoptosis after SCI (see Fig. 2), we expected that ghrelin treatment would improve functional recovery after injury. Functional recovery was then evaluated for 35 d after injury using the BBB rating scale (34), inclined plane test (35), grid walk test (36), and footprint analysis (37, 59). As shown in Fig. 7A, ghrelin treatment (40, 80, or 160  $\mu$ g/kg) significantly increased hindlimb locomotor function, as assessed by BBB scores, 21–35 d after injury compared with that observed in vehicle-treated control. The angles of incline determined 1–4 wk after injury were also significantly higher in ghrelin-treated (40, 80, or 160  $\mu$ g/kg) groups compared with those in vehicle-treated control group (Fig. 7B). The ability to control and place the hindlimbs precisely was tested on a horizontal grid at 35 d after injury. As shown in Fig. 7C, the number of mistakes (footfalls on the grid walk) in ghrelin-treated (40, 80, or 160  $\mu$ g/kg) groups was significantly lower than that observed in the vehicle-treated group. Footprint analyses at 35 d after SCI reveal that the ghrelin-treated (40, 80, or 160



**FIG. 5.** Ghrelin increases BDNF expression and decreases proNGF expression after SCI. Total mRNA and protein from spinal cord tissues were prepared, and RT-PCR and Western blot analysis were performed as described in *Materials and Methods* ( $n = 3$ ). The gels presented are representative of results from three separate experiments. BDNF mRNA (A) and protein (B) were increased and peaked at 1 d after SCI. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. C, Representative photographs of fluorescence microscopy show neurons (NeuN) and oligodendrocytes (CC1) expressed BDNF (arrows), whereas astrocytes (GFAP) and microglia (OX-42) were negative at 1 d after injury. Scale bar, 30  $\mu\text{m}$ . D and E, Ghrelin treatment (80  $\mu\text{g}/\text{kg}$ ;  $n = 3$ ) significantly increased the level of BDNF mRNA and protein expression at 1 d after injury when compared with that in vehicle control. Values are means  $\pm$  SD of three separate experiments. \*,  $P < 0.05$ . F, The MEK/ERK inhibitor, PD98059 (30  $\mu\text{mol}$ ), was injected directly into the spinal cord at the lesion epicenter after injury. PD98059 treatment significantly decreased the levels of both ERK and BDNF expression at 1 d after injury when compared with that in vehicle-treated rats. Values are means  $\pm$  SD of three separate experiments. \*,  $P < 0.05$ . G, Ghrelin treatment significantly decreased the level of proNGF expression when compared with that observed in vehicle-treated control at 5 d after injury. Values are means  $\pm$  SD of three separate experiments. \*,  $P < 0.01$ .

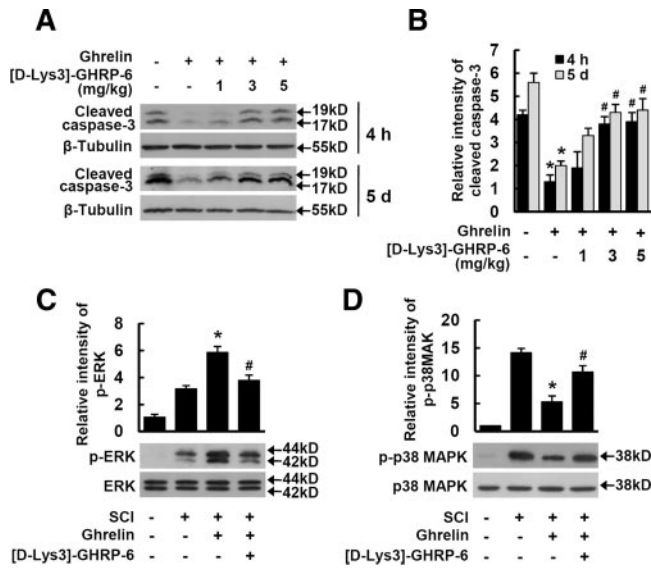
$\mu\text{g}/\text{kg}$ ) rats showed fairly consistent plantar stepping weight support and very few toe drags (Fig. 7D). By contrast, vehicle-treated rats showed inconsistent dorsal stepping and extensive drags as revealed by ink streaks extending from both hindlimbs (Fig. 7D). Because of the hindlimb dragging in the vehicle-treated animals (Fig. 7D), we were not able to quantify footprint analysis using toe spread and ipsilateral distances (limb coordination). Finally, injection of the specific ghrelin receptor antagonist, [D-Lys3]-GHRP-6 (3 mg/kg), immediately after ghrelin (80  $\mu\text{g}/\text{kg}$ ) treatment significantly abolished the beneficial effect of ghrelin on BBB scores at 14–35 d after injury (Fig. 7E).

### Ghrelin reduces axon and myelin loss and lesion size after SCI

The functional deficit after SCI is known to be correlated with the massive loss of axons in the WM (60). Because ghrelin treatment improved functional recovery af-

ter SCI (see Fig. 7), we expected that ghrelin treatment would reduce axon loss. We first performed immunostaining with antibodies against 200-kDa neurofilament protein (NF200) and 5-hydroxytryptamine (5-HT) to detect the remaining axons using spinal tissues recovered 38 d after injury. In sham controls, NF200-positive axons in the ventral and dorsolateral funiculus were dense and axonal packing was uniform (Supplemental Fig. 3, A and B, sham). By contrast, in injured tissues, the density of axons was markedly decreased and exhibited a patchy distribution (Supplemental Fig. 3, A and B, vehicle). However, the number of NF200-positive axons in the ventral and dorsolateral funiculus was significantly higher in the ghrelin-treated group compared with those in the vehicle-treated control group (Supplemental Fig. 3C). Next, to assess the extent of spared descending axonal pathways reaching the site of injury, fluorogold (FG) was injected into the region caudal (L1) to the lesion site at 38 d after injury, and FG-labeled neurons in selected supraspinal nuclei were counted. As shown in Supplemental

Fig. 3, D and E, the numbers of FG-labeled neurons in red nucleus, pontine reticular nuclei, lateral vestibular nucleus, and ventral dorsal medullary reticular fields were significantly higher in ghrelin-treated groups than those in vehicle-treated groups. These results indicate that ghrelin treatment reduces the extent of axon loss after injury. Histological analyses also revealed that the lesion volume of ghrelin-treated groups at 38 d after injury was significantly smaller than that of the vehicle-treated control (Supplemental Fig. 4, A and B). Next, the extent of myelin loss after injury was assessed by Luxol-fast blue staining. As shown in Supplemental Fig. 4, C–H, extensive myelin loss near the lesion area was evident in the vehicle-treated group at 38 d after injury compared with the sham control (Supplemental Fig. 4, D and G), whereas ghrelin treatment apparently attenuated myelin loss (Supplemental Fig. 4, E and H). Myelin integrity was also evaluated by immunostaining using an anti-MBP antibody. Immunostaining re-

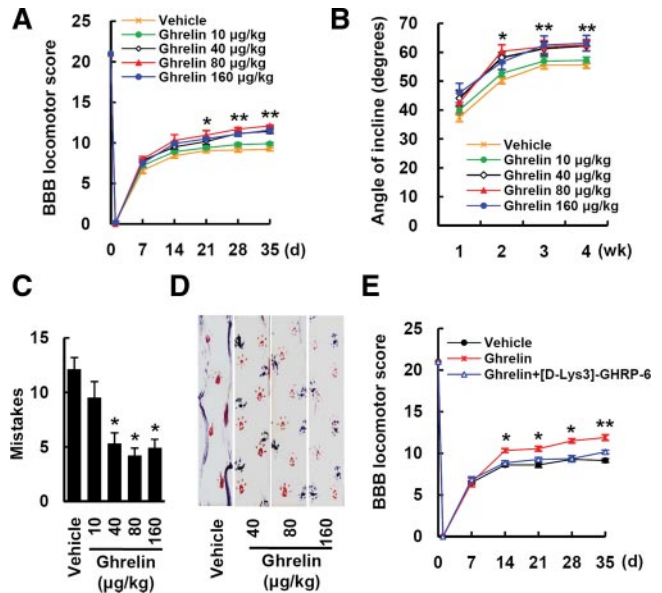


**FIG. 6.** Effects of ghrelin and ghrelin receptor antagonist on the activation of caspase-3, ERK, and p38MAPK after SCI. The ghrelin receptor (GHS-R1a) antagonist, [D-Lys3]-GHRP-6 (1, 3, and 5 mg/kg), was injected before ghrelin treatment after SCI, and total lysates were prepared as described in *Materials and Methods* (n = 3). **A**, Western blottings of activated caspase-3 at 4 h and 5 d after injury. **B**, Quantitative analysis of Western blottings shows that [D-Lys3]-GHRP-6 (3 and 5 mg/kg) treatment significantly reduced the inhibitory effect of ghrelin on caspase-3 activation after injury. Values are means ± SD of three separate experiments. \*, P < 0.05 compared with vehicle treatment; #, P < 0.05 compared with ghrelin treatment. **C**, Western blottings of p-ERK at 12 h after injury. Quantitative analysis of Western blottings shows that [D-Lys3]-GHRP-6 (3 mg/kg) treatment significantly alleviated the stimulatory effect of ghrelin on p-ERK after injury. Values are means ± SD of three separate experiments. \*, P < 0.05 compared with vehicle treatment; #, P < 0.05 compared with ghrelin treatment. **D**, Western blottings of p-p38MAPK at 5 d after injury. Quantitative analysis of Western blottings shows that [D-Lys3]-GHRP-6 (3 mg/kg) treatment significantly mitigated the inhibitory effect of ghrelin on p-p38MAPK after injury. Values are means ± SD of three separate experiments. \*, P < 0.05 compared with vehicle; #, P < 0.05 compared with ghrelin treated rats.

vealed that the intensity of MBP expression was higher in the ghrelin-treated rats than that in the vehicle-treated rats at 38 d after injury (Supplemental Fig. 4I). Furthermore, Western blot analysis revealed that the level of MBP (14 kDa) was markedly decreased at 39 d after injury compared with sham controls as previously reported (Supplemental Fig. 4J) (61). However, the level of MBP was significantly higher in the ghrelin-treated groups when compared with the vehicle-treated groups at 42 d after injury (Supplemental Fig. 4J). These observations indicate that ghrelin treatment reduces the extent of myelin loss after injury.

**Determination of therapeutic time windows for administration of ghrelin after SCI**

The time window for administration of a neuroprotective compound in acute SCI is a critical factor with respect to its possible therapeutic use. To determine ghrelin’s ther-



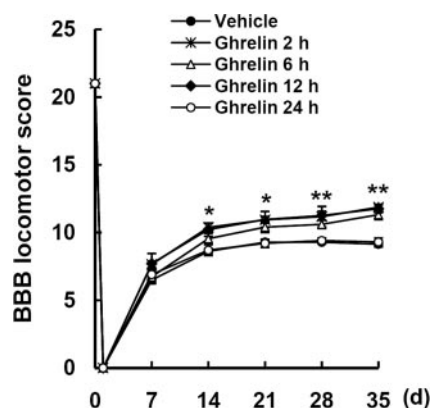
**FIG. 7.** Ghrelin improves functional recovery after SCI. Ghrelin (10, 40, 80, and 160 μg/kg) was administered immediately after injury and then further treated every 6 h for 1 d. Functional recovery was assessed by BBB test, inclined plane test, grid walk test, and footprint analysis. Each value represents the mean ± SD obtained from 20 animals. **A**, BBB scores of vehicle- and ghrelin-treated groups after injury. \*, P < 0.05; \*\*, P < 0.01. **B**, Inclined plane test of vehicle- and ghrelin-treated groups after injury. \*, P < 0.05; \*\*, P < 0.01. **C**, Grid walk test of vehicle- and ghrelin-treated groups at 35 d after injury. \*, P < 0.05. **D**, Representative footprints obtained from each group at 35 d after SCI show that ghrelin-treated rat showed fairly consistent weight support plantar stepping and very little toe drags. By contrast, vehicle-treated animals showed consistent dorsal stepping and extensive drags. **E**, [D-Lys3]-GHRP-6 (3 mg/kg) were administered immediately before ghrelin (80 μg/kg) treatment after injury and functional recovery by BBB test was performed as described in *Materials and Methods* (n = 20). Note that [D-Lys3]-GHRP-6 treatment alleviated the beneficial effect of ghrelin on BBB scores. \*, P < 0.05; \*\*, P < 0.01.

apeutic time window, ghrelin (80 μg/kg) was injected at 2, 6, 12, or 24 h after injury and then at the same dose every 6 h for an additional day; the locomotor function was assessed with the BBB scale. As shown in Fig. 8, ghrelin treatment significantly improved BBB scores when it was injected at 2, 6, and 12 h after injury as compared with those of the vehicle-treated control. However, when ghrelin administration was delayed until 24 h after injury, the BBB scores were not significantly different from those of the vehicle-treated control (Fig. 8).

**Discussion**

We demonstrated for the first time that after moderate contusion injury to the rat spinal cord, postinjury treatment with ghrelin improved functional recovery by inhibiting apoptotic cell death of neurons and oligodendrocytes. We also showed that the antiapoptotic effect of ghrelin on neurons and oligodendrocytes may be mediated





**FIG. 8.** Delayed administrations of ghrelin improve functional recovery after SCI. After SCI, ghrelin (80  $\mu\text{g}/\text{kg}$ ) was administered at 2, 6, 12, or 24 h after injury and then administered the same dose of ghrelin every 6 h for 1 d, and functional recovery was assessed by BBB test ( $n = 20$ ). Ghrelin treatment (given at 2, 6, and 12 h after injury) significantly improved locomotor function as assessed by BBB score when compared with vehicle. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ .

in part by increasing ERK-dependent BDNF expression after injury. Furthermore, we showed that the antiapoptotic effect of ghrelin on oligodendrocytes may be mediated in part by decreasing p38MAPK-dependent proNGF expression after injury. Finally, we demonstrated that the antiapoptotic effect of ghrelin was mediated through the ghrelin receptor, and that time of administration after injury played a key role in beneficial results.

In the present study, ghrelin (40, 80, or 160  $\mu\text{g}/\text{kg}$ ) was administered after injury and then was further injected every 6 h for 1 d with a same dose of ghrelin. It has been known that a high dose of ghrelin (4 mg/kg  $\cdot$  d for 2 wk) can induce adiposity in mice (13). However, we ignored the possibility of the adiposity induced by ghrelin, because we used a very low concentration of ghrelin (0.05–0.8 mg/kg  $\cdot$  d for 1 d) in this study. In fact, there were no significant changes in body weight among the experimental groups during the experiment (data not shown). Also, neither significant side effects nor an increase in mortality by ghrelin treatment were observed. Furthermore, ghrelin (80 or 160  $\mu\text{g}/\text{kg}$ ) treatment similar to dosage used in this study was known to decrease infarct volume in focal ischemia and improve motor function by attenuating MPTP-induced dopaminergic neuronal loss (25, 28).

Our results indicate that the antiapoptotic effect of ghrelin might be mediated through the mitochondrial-dependent apoptotic pathway, because ghrelin treatment inhibited cytochrome *c* release and caspase-3 activation (see Fig. 3). Recent reports showed that the mechanism of the neuroprotective effect of ghrelin may be related to attenuating the activity of caspase-3 via increasing Bcl-2/Bax ratio in MPTP-induced Parkinson's disease (PD) animal model (27) and ischemic neuronal injury *in vitro* [Chung *et al.* (25)]. However, our study showed that ghrelin

treatment did not change Bcl-2/Bax ratio after injury (Supplemental Fig. 1, C and D). Further study is required to elucidate the mechanism underlying ghrelin-mediated inhibition of cytochrome *c* release and caspase-3 activation after SCI.

It has been shown that ERK activation is involved in neuronal survival after SCI (31, 62). In the present study, we showed that ghrelin treatment increased ERK activation (see Fig. 4). Also, BDNF has been well known to involve in cell survival and functional recovery after SCI (28, 29, 49, 54). Here, we demonstrated that treatment with PD98059, an inhibitor of MEK/ERK, inhibited BDNF expression, suggesting that BDNF production may be mediated by the activation of ERK pathway (see Fig. 5F). These results suggest that the antiapoptotic effect of ghrelin on neurons and oligodendrocytes after SCI may be mediated in part by increasing BDNF expression via ERK signaling. It should be pointed out that the transcription factors involved in the BDNF expression after injury were not examined in the present study.

We demonstrated by double-labeling immunohistochemistry that after injury, neurons and oligodendrocytes were positive for BDNF, whereas astrocytes and microglia were negative for BDNF (see Fig. 5C). However, several reports show that glial cells also express BDNF after SCI. For example, Hayashi *et al.* (49) showed that by *in situ* hybridization, astrocytes express BDNF mRNA after injury. Ikeda *et al.* (50) also demonstrated that by double-staining with *in situ* hybridization and immunohistochemistry, neurons, astrocytes, and macrophage/microglia express BDNF after injury. The discrepancy in the cell types expressing BDNF may be attributable to the type of injury and the detection method. Although we were not able to observe BDNF-positive astrocytes or microglia in the injured spinal cord, we cannot rule out the possibility that low levels of BDNF may be expressed by glial cells after injury.

The present study showed that ghrelin treatment after SCI inhibited the activation of p38MAPK and proNGF expression (see Figs. 4 and 5). Our previous report also demonstrates that proNGF production after SCI via activation of p38MAPK in microglia is involved in oligodendrocyte cell death (8). Thus, our data suggest that the antiapoptotic effect of ghrelin on oligodendrocytes may be mediated in part by decreasing proNGF production via inhibition of p38MAPK activation in microglia after injury. Furthermore, we showed that the inhibition of p38MAPK activation by ghrelin was mediated through ghrelin receptor (see Fig. 6). However, our immunohistochemical results revealed that ghrelin receptor was not expressed in microglia (data not shown). Thus, the mechanism underlying ghrelin-mediated inhibition of

p38MAPK activation and proNGF production in microglia remains to be determined.

Recent evidence indicates that ghrelin inhibits inflammatory responses and oxidative stress *in vivo* and *in vitro*. For example, ghrelin inhibits TNF- $\alpha$  and IL-6 expression and nitric oxide production in peritoneal macrophages, human umbilical vein endothelial cells, and in arthritic rats (63, 64). Ghrelin also reduces malondialdehyde levels and myeloperoxidase activity and increases the level of glutathione in alendronate-induced oxidative gastric damage in the rat (65). A recent report also shows that ghrelin inhibits neuronal apoptosis by inhibiting microglial activation in MPTP-induced PD animal model (28). In addition, our preliminary results revealed that ghrelin treatment significantly decreased the number of activated microglia after SCI (data not shown). Furthermore, expression of proinflammatory cytokines and mediators, such as TNF- $\alpha$ , IL-1 $\beta$ , cyclooxygenase-2, and inducible nitric oxide synthase, was decreased by ghrelin treatment after injury (data not shown). These preliminary results indicate that the neuroprotective effect of ghrelin after SCI may be mediated in part by attenuating inflammatory responses and oxidative stress in microglia.

Finally, we also showed that the level of *IGF-I* mRNA expression was increased by ghrelin treatment after SCI (Supplemental Fig. 2). It has been known that IGF-I is involved with neuronal survival and improves functional recovery after SCI (56–58). These findings taken together demonstrate that it is likely that the cellular and molecular mechanisms underlying ghrelin's neuroprotection after SCI appear to be multifaceted.

Endocrine activities of ghrelin are known to be mediated by GHS-R1a, a G protein-coupled receptor (14, 23). GHS-R1a is expressed mainly in the pituitary and hypothalamus and also present extensively in central and peripheral tissues (14, 15). Recent reports show that the neuroprotective effect of ghrelin is mediated by GHS-R1a in glucose-oxygen-induced apoptosis in hypothalamic neuron culture and in an MPTP-induced PD animal model (25, 26). These observations are in agreement with our findings that the neuroprotective effect of ghrelin after SCI was mediated through the ghrelin receptor. Furthermore, we demonstrated that both spinal cord neurons and oligodendrocytes expressed the ghrelin receptor. However, the physiological function of ghrelin receptors on these cells requires further investigation.

A significant sparing of axons within the ventral and dorsolateral funiculus is known to contribute to locomotor control (66). Our data demonstrated that the density of spared axons within the ventral and dorsolateral funiculus was higher in the ghrelin-treated group than those observed in the vehicle control group (see Supplemental

Fig. 3, A and B). Furthermore, the number of 5-HT-positive serotonergic fibers in the ventral horn was higher in the ghrelin treatment group than that in the vehicle control group (see Supplemental Fig. 3C). Because 5-HT is one of the key neurotransmitters responsible for initiating locomotion (67), these serotonergic fibers are correlated with locomotor function. The preservation of axons by ghrelin was also confirmed by FG retrograde labeling (see Supplemental Fig. 3, D and E). The conservation of axons influences the results of the inclined plane scores shown in Fig. 7B, because the integrity of the nonpyramidal tracts, in particular the rubrospinal tracts, vestibulospinal tracts, and raphespinal tracts, is highly correlated with inclined plane performance in spinal cord injured rats (68). The grid walk scores indicate deficits in the descending pathways for fine motor control after SCI (69, 70). Figure 7C shows that the number of footfalls (mistakes) in the ghrelin-treated groups was lower than that of vehicle control, suggesting that fine motor control was significantly improved by ghrelin treatment.

Methylprednisolone, which is currently used for treatment of acute SCI in humans, has shown no beneficial effect in SCI patients if not initiated early after injury (71, 72). Therefore, the determination of the therapeutic time windows for candidate agents is pivotal to judge the possibility for therapeutic use after SCI. Here, our data show that ghrelin significantly improved functional recovery when administered at 2, 6, and 12 h after injury (see Fig. 8). These results suggest that ghrelin can be potentially useful as a therapeutic agent for acute SCI.

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