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Alcohol exposure induces depression-like behavior by decreasing hippocampal neuronal proliferation through inhibition of the BDNF-ERK pathway in gerbils

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Depression is one of the most prevalent diseases of alcohol abuse. Brain-derived neurotrophic factor (BDNF) plays a critical role in cell survival in the hippocampus. Phosphorylation of extracellular signal-regulated kinase 1/2 (p-ERK1/2) is induced by BDNF, and it regulates cell proliferation and differentiation in the brain. We investigated the effects of alcohol intake on depression-like behavior, cell proliferation, expressions of BDNF and its downstream molecules in the hippocampus using Mongolian gerbils. The gerbils were divided into four groups: control group, 0.5 g/kg alcohol-treated group, 1 g/kg alcohol-treated group, 2 g/kg alcohol-treated group. Each dose of alcohol was orally administered for 3 weeks. The present results demonstrated that alcohol intake induced depression-like behavior. Both 5-hydroxytryptamine synthesis and its synthesizing enzyme tryptophan hydroxylase expression in the dorsal raphe and cell proliferation in the hippocampal dentate gyrus were decreased by alcohol intake. Alcohol intake suppressed BDNF expression, and resulted in the decrease of its downstream molecules, pERK1/2 and Bcl-2, in the hippocampus. We showed that alcohol intake may lead to a depressed-like state with reduced hippocampal cell proliferation through inhibition of the BDNF-ERK signaling pathway.

Keywords: alcohol; depression; cell proliferation; brain-derived neurotrophic factor; extracellular signal-regulated kinase 1/2

Introduction

Alcohol dependence or abuse is one of the most costly healthcare problems worldwide. Alcohol toxicity and degeneration target the liver primarily, with the brain as the second major target. Alcohol addiction is known to cause a variety of neuropsychiatric disorders, resulting in cognitive decline and dementia (de la Monte et al., 2009; Rubio et al., 2011). Of these disorders, depression is one of the most prevalent psychiatric diseases of alcohol abuse (Rubio et al., 2011).

Depression is a persistently and abnormally decreased mood state with disturbed cognitive and physical functions. Depression is known to be closely associated with decreased release of serotonergic neurotransmitters, such as 5-hydroxytryptamine (5-HT, serotonin). Antidepressant drugs reduce symptoms of depression through an increase of 5-HT level in the dorsal raphe (Sellers et al., 1992; Kim et al., 2002). Tryptophan hydroxylase (TPH) catalyzes the rate-limiting step of 5-HT biosynthesis in the serotonergic neurons (Chamas et al., 1999); therefore the expression of 5-HT and TPH has been used as the markers of the depression.

Recent studies emphasized the histological and cell biological alterations of the hippocampus in depression (Moonat et al., 2010; Morris et al., 2010). Alcohol intake induced depression with reduced neurogenesis in the hippocampus (Nixon, 2006; Morris et al., 2010). The hippocampal dentate gyrus is the brain region that continually generates new neurons throughout life in rodents, primates, and humans (Eriksson et al., 1998; Nixon and Crews, 2004). The functional role of newborn neurons remains unclear; however, newly generated neurons are known to contribute to the improvement of various brain diseases, including depression (Duman, 2004). Proliferation of hippocampal progenitor cells and synaptic plasticity were reduced in animal models of depression and treatment with antidepressants ameliorated depression-like behavior with increased hippocampal proliferation (Malberg and Duman, 2003; Santarelli et al., 2003; Stevenson et al., 2009).

Depression is closely linked to the expression of several neurotrophins (Altar, 1999; Angelucci et al., 2005). Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, has an important role in the proliferation, differentiation, and growth of the hippocampal progenital cells at the developmental stage (Huang and Reichardt. 2001; Lee and Son, 2009). BDNF controls neuronal survival and plasticity through binding to the high-affinity receptor tyrosine kinase B (TrkB) (Givalois et al., 2001). The BDNF-

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TrkB interaction promotes the survival and differentiation of neurons, and this BDNF-TrkB interaction is also involved in learning ability and memory function (Koponen et al., 2004; Pietropaolo et al., 2007).

BDNF activates the mitogen-activated protein kinases (MAPK) pathway by binding to the receptor TrkB (Moonat et al., 2010). MAPK are serine/threonine-specific protein kinases that respond to extracellular stimuli and their pathways include many proteins, such as Ras, Raf, MAP kinase kinase (MEK), and extracellular signal-regulated kinase 1/2 (ERK1/2). ERK1/2 that is phosphorylated by BDNF regulates various cellular activities, including proliferation and cell survival. BDNF was reduced in alcohol-induced depression in animals (Stevenson et al., 2009; Moonat et al., 2010). Antidepressants improved functional problems in depression by increasing BDNF expression (Angelucci et al., 2005). BDNF can be considered as a therapeutic substrate for alcohol-induced depression.

The main pathological finding of depression is suppression of 5-HT and depression also accompanies a decrease in cell proliferation and survival in the hippocampus. It is well established that alcohol induces depression-like behavior and alcohol intake inhibits BDNF expression in the brain; however, the interactions of alcohol intake on the depression-like behavior in relation to the BDNF-ERK signaling pathway in the hippocampus are largely unknown. We investigated the effects of alcohol intake on depression-like behavior, cell proliferation, and expressions of BDNF and their downstream molecules in the hippocampus using Mongolian gerbils. Depression-like behavior was evaluated by a forced swimming test (FST). New cell proliferation and expressions of 5-HT and TPH were detected by immunohistochemistry for 5-bromo-2′-deoxyuridine (BrdU), 5-HT, and TPH.

Materials and methods

Experimental animals and treatments

Male Mongolian gerbils weighing 70 ± 10 g (6 months old) were used in the present study. The experimental procedures were performed in accordance with the animal care guidelines of the National Institutes of Health (NIH) and the Korean Academy of Medical Sciences. The animals were housed under controlled temperature (23 ± 2°C) and lighting (08:00 to 20:00 h) conditions with food and water available *ad libitum*. The animals were randomly divided into four groups ($n = 10$ in each group): the control group, the 0.5 g/kg alcohol-treated group, the 1 g/kg alcohol-treated group, and the 2 g/kg alcohol-treated group. Alcohol was administered orally, once a day for 3 weeks. BrdU (50 mg/kg) was intraperitoneally injected into all animals twice a week for 3 weeks. Each animal was sacrificed 24 h after the last alcohol administration.

Forced swimming test

The FST was conducted according to the previously described method (Sung et al., 2010). Experimental animals were dropped individually into glass cylinders. The glass cylinder (height 50 cm, diameter 15 cm) contained water at a temperature of 27°C. The water depth was 30 cm. All of the experimental gerbils underwent a pre-test for 15 min to eliminate the acute stress of the water and to provide the animals with the ability to adapt to the water. Twenty-four hours after pre-test, the animals were tested for 5 min. During the test session, the climbing time and the immobility time were analyzed using a Smart version 2.5 video tracking system (Panlab, Barcelona, Spain). Climbing behavior consisted of upward-directed movements of the fore-paws along the side of the swimming chamber. Immobility behavior was defined to occur when no additional activity was observed other than the actions needed to keep the animals' head above the water.

Tissue preparation for immunohistochemistry

Tissue preparation was done according to the previously described method (Kim et al., 2010b). The experimental animals were fully anesthetized using Zoletil 50® (10 mg/kg, i.p.; Vibac Laboratories, Carros, France), transcardially perfused with 50 mM phosphate-buffered saline (PBS), and fixed with a freshly prepared solution consisting of 4% paraformaldehyde in 100 mM phosphate buffer (PB, pH 7.4). Brains were dissected, post-fixed in the same fixative overnight, and transferred to 30% sucrose for cryoprotection. Coronal sections of 40 μm thickness were made with a freezing microtome (Leica, Nussloch, Germany). Ten slice sections on average in the hippocampus and dorsal raphe were collected from each animal.

BrdU immunohistochemistry

BrdU-specific immunohistochemistry was performed by the previously described method (Kim et al., 2010a). Briefly, the sections were permeabilized by incubation in 0.5% Triton X-100 in PBS for 20 min, incubated in 50% formamide-2 × standard saline citrate (SSC) at 65°C for 2 h, denatured in 2 N HCl at 37°C for 30 min, and rinsed twice in 100 mM sodium borate (pH 8.5). The sections were then incubated overnight at 4°C with BrdU-specific mouse monoclonal antibody (1:600; Roche, Mannheim, Germany). The sections were washed three times with PBS and incubated for 1 h with biotinylated mouse secondary antibody (1:200;

Vector Laboratories, Burlingame, CA, USA). The sections were incubated for an additional 1 h with avidin-biotin horseradish peroxidase complex (1:100; Vector Laboratories). For visualization, the sections were incubated for 5 min in 50 mM Tris-HCl (pH 7.6) containing 0.03% 3,3′-diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, MO, USA), 40 mg/mL nickel chloride, and 0.03% H_2O_2. The sections were then mounted on gelatin-coated glass slides.

Immunohistochemistry for 5-HT and TPH

Immunohistochemistry for 5-HT and TPH was performed by the previously described method (Seo et al., 2011). Briefly, the sections were incubated in PBS for 10 min and washed three times, again with PBS, and then incubated in 1% H_2O_2 for 30 min. Next, the sections were incubated overnight with rabbit anti-5-HT antibody (Immunostar, Hudson, NY, USA) at a dilution of 1:500 for visualization of 5-HT expression or with mouse anti-TPH antibody (Oncogene Research Product, Cambridge, UK) at a dilution of 1:1000 for visualization of TPH expression. The sections were then incubated for 1 h with biotinylated anti-rabbit secondary antibody or with anti-mouse secondary antibody (Vector Laboratories). The sections were subsequently incubated with avidin-biotin-peroxidase complex (Vector Laboratories) for 1 h at room temperature. Immunoreactivity was visualized by incubating the sections in a solution consisting of 0.05% DAB (Sigma Chemical Co.) and 0.01% H_2O_2 in 50 mM Tris buffer (pH 7.6) for approximately 3 min. The sections were then mounted on gelatin-coated glass slides.

Western blot analysis

Western blot was performed by the previously described method (Kim et al., 2010a). The hippocampal tissues were homogenized on ice, and lysed in a lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 1 mM EGTA, 1.5 mM $MgCl_2 \cdot 6H_2O$, 1 mM sodium orthovanadate, and 100 mM sodium fluoride. Protein content was measured using a Bio-Rad colorimetric protein assay kit (Hercules, CA, USA). Protein (30 μg) was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. Mouse β-actin antibody (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit BDNF antibody (1:1000; Santa Cruz Biotechnology), rabbit TrkB antibody (1:1000; Santa Cruz Biotechnology), mouse Bcl-2 antibody (1:1000; Santa Cruz Biotechnology), mouse p-ERK1/2 antibody (1:1000; Santa Cruz Biotechnology), and rabbit t-ERK1/2 antibody (1:1500, Vector Laboratories) were used as the primary antibodies. Horseradish peroxidase-conjugated anti-rabbit antibodies for BDNF, t-ERK1/2, and TrkB (1:5000; Vector Laboratories) and horseradish peroxidase-conjugated anti-mouse antibodies for β-actin, Bcl-2, and p-ERK1/2 (1:3000; Amersham Pharmacia Biotech GmbH, Freiburg, Germany) were used as the secondary antibodies.

Experiments were performed in normal laboratory conditions and at room temperature, except for the transferred membranes. Transferred membranes were examined at 4°C with the cold pack and pre-chilled buffer. Band detection was performed using an enhanced chemiluminescence (ECL) detection kit (Santa Cruz Biotechnology).

Data analysis

The area of the granular layer of the dentate gyrus was measured using an Image-Pro Plus image analyzer (Media Cybernetics Inc., Silver Spring, MD, USA). The number of BrdU-positive cells was counted hemilaterally and expressed as the number of cells per mm^2 of the cross-sectional area of the granular layer of the dentate gyrus. The numbers of 5-HT-positive and TPH-positive cells were counted and expressed as the number of cells per mm^2 of the cross-sectional area of the dorsal raphe. To compare the relative expression of proteins, the detected bands were calculated densitometrically using Molecular Analyst™, version 1.4.1 (Bio-Rad).

All data were analyzed using SPSS statistical software (version 12.0; SPSS Inc., Chicago, IL, USA). The data were expressed as the mean ± standard error of the mean (SEM). For comparisons among the groups, one-way ANOVA and Duncan's post-hoc test were performed, and differences were considered statistically significant at $P < 0.05$.

Results

Effect of alcohol treatment on depression-like behavior in the FST

The climbing time was 161.52 ± 3.76 sec in the control group, 126.89 ± 12.54 sec in the 0.5 g/kg alcohol-treated group, 93.34 ± 13.46 sec in the 1.0 g/kg alcohol-treated group, and 87.63 ± 12.19 sec in the 2.0 g/kg alcohol-treated group (Figure 1, left). The immobility time was 82.98 ± 0.61 sec in the control group, 116.11 ± 17.34 sec in the 0.5 g/kg alcohol-treated group, 148.45 ± 8.05 sec in the 1.0 g/kg alcohol-treated group, and 161.75 ± 6.68 sec in the 2.0 g/kg alcohol-treated group (Figure 1, right).

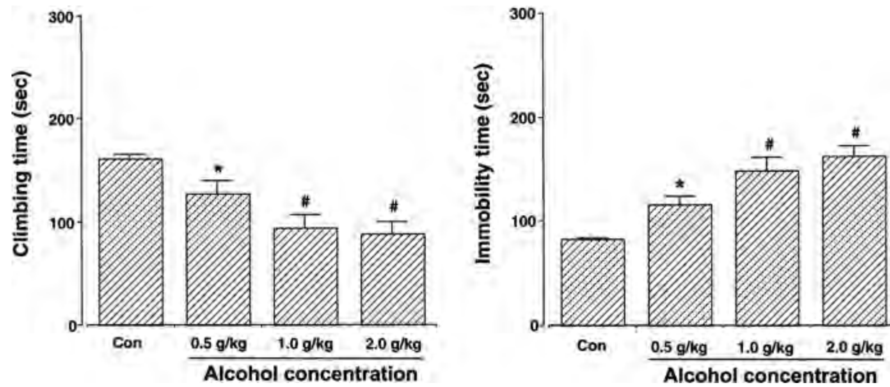

Figure 1. Effect of alcohol intake on forced swimming test. * represents $P < 0.05$ compared to the control group. # represents $P < 0.05$ compared to the 0.5 g/kg alcohol-treated group. Con = control group.

Effect of alcohol treatment on 5-HT synthesis and TPH expression in the dorsal raphe

The number of 5-HT-positive cells in the dorsal raphe was 161.17 ± 8.80 in the control group, 117.17 ± 6.78 in the 0.5 g/kg alcohol-treated group, 65.83 ± 3.84 in the 1.0 g/kg alcohol-treated group, and 53.33 ± 4.89 in the 2.0 g/kg alcohol-treated group (Figure 2A). The number of TPH-positive cells in the dorsal raphe was 132.75 ± 8.10 in the control group, 81.50 ± 4.93 in the 0.5 g/kg alcohol-treated group, 56.13 ± 6.48 in the 1.0 g/kg alcohol-treated group, and 51.38 ± 6.59 in the 2.0 g/kg alcohol-treated group (Figure 2B).

Effect of alcohol treatment on cell proliferation in the hippocampal dentate gyrus

The number of BrdU-positive cells in the hippocampal dentate gyrus was $148.19 \pm 14.52/mm^2$ in the control group, $100.60 \pm 6.14/mm^2$ in the 0.5 g/kg alcohol-treated group, $90.84 \pm 9.69/mm^2$ in the 1.0 g/kg alcohol-treated group, and $57.20 \pm 10.12/mm^2$ in the 2.0 g/kg alcohol-treated group (Figure 3).

Effect of alcohol treatment on BDNF signaling molecules in the hippocampus

We analyzed the relative expression of BDNF, TrkB, p-ERK1/2, and Bcl-2 proteins (Figure 4A). When the level of BDNF in the control group was set at 1.00, the level of BDNF was 0.73 ± 0.10 in the 0.5 g/kg alcohol-treated group, 0.28 ± 0.04 in the 1.0 g/kg alcohol-treated group, and 0.24 ± 0.06 in the 2.0 g/kg alcohol-treated group (Figure 4B). When the level of TrkB in the control group was set at 1.00, the level of TrkB was 0.53 ± 0.05 in the 0.5 g/kg alcohol-treated group, 0.50 ± 0.02 in the 1.0 g/kg alcohol-treated group, and 0.46 ± 0.06 in the 2.0 g/kg alcohol-treated group (Figure 4C). When the level of p-ERK1/2 in the control group was set at 1.00, the level of p-ERK1/2 was 0.71 ± 0.08 in the 0.5 g/kg alcohol-treated group, 0.61 ± 0.07 in the 1.0 g/kg alcohol-treated group, and 0.53 ± 0.08 in the 2.0 g/kg alcohol-treated group (Figure 4D). When the level of Bcl-2 in the control group was set at 1.00, the level of Bcl-2 was 0.78 ± 0.01 in the 0.5 g/kg alcohol-treated group, 0.57 ± 0.08 in the 1.0 g/kg alcohol-treated group, and 0.51 ± 0.09 in the 2.0 g/kg alcohol-treated group (Figure 4E).

Discussion

In the present results, the climbing time was decreased and the immobility time was increased with increasing alcohol administration. The behavioral parameters, such as the time of climbing and immobility, in the FST are interpreted as an indicator determining depression (Detke et al., 1995). Increase in immobility is interpreted as a failure of persistence in escape-directed behavior (Cryan et al., 2002). Alcohol dependence and/or abstinence induced depression-like symptoms by showing an increase in immobility time and a decrease in climbing time in the FST (Stevenson et al., 2009). Decrease in climbing time and increase in immobility time were observed in maternal rats separated from their pups, representing depression-like behavior (Sung et al., 2010). In this study, the gerbils that received alcohol showed depression-like behavior.

In the present results, 5-HT synthesis and TPH expression in the dorsal raphe were decreased with increasing alcohol administration. About 50% of neurons in the dorsal raphe contain 5-HT (Arvidsson et al., 1994). Sellers et al. (1992) showed that the 5-HT concentration in the dorsal raphe was significantly lower in rats with alcohol-induced depression than in normal rats. Reduced activity of the brain 5-HT system

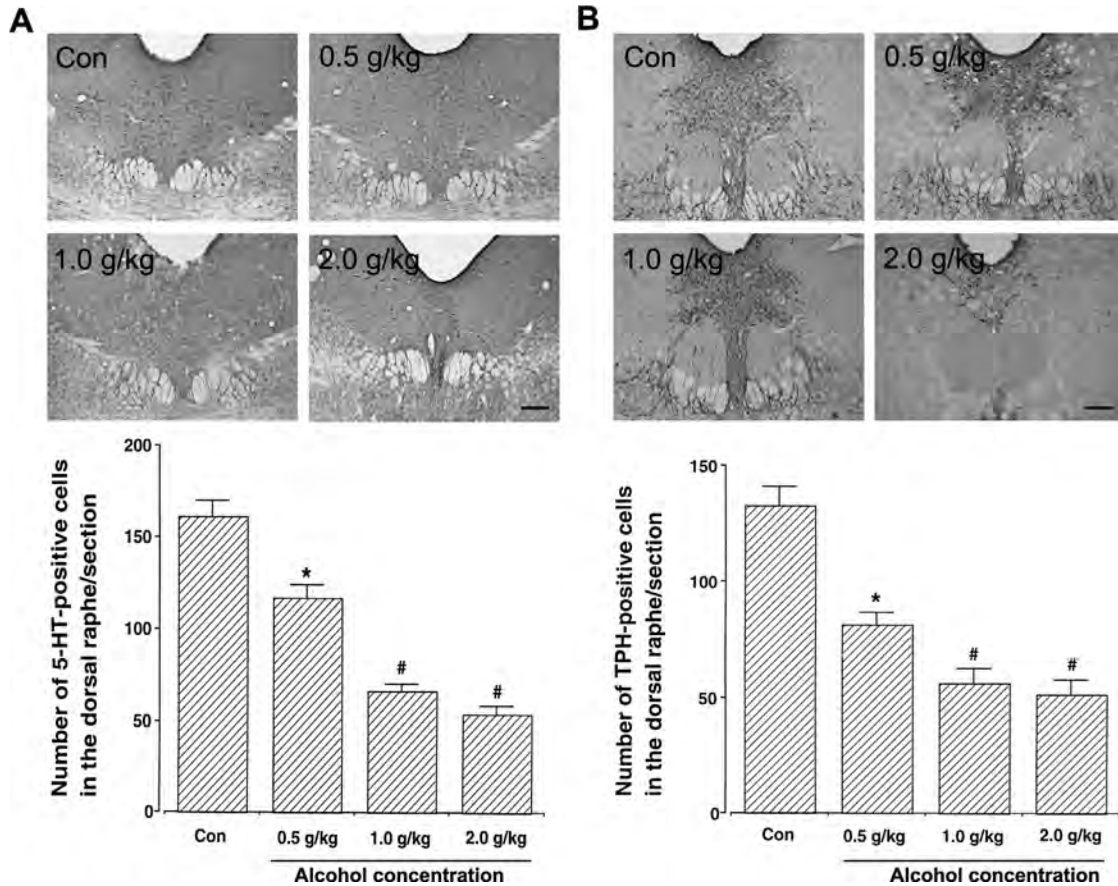

Figure 2. Effect of alcohol intake on 5-hydroxytryptamine (5-HT) synthesis (A) and tryptophan hydroxylase (TPH) expression (B) in the dorsal raphe. Upper panel: photomicrographs of 5-HT and TPH-positive cells in the dorsal raphe. The scale bar represents 800 μm. Lower panel: numbers of 5-HT and TPH-positive cells in each group. * represents $P < 0.05$ compared to the control group. # represents $P < 0.05$ compared to the 0.5 g/kg alcohol-treated group. Con = control group.

is highly correlated with the pathophysiology of depression (Kim et al., 2002; Yang et al., 2008; Vasudeva et al., 2011). As TPH is an enzyme that regulates the synthesis of 5-HT, its expression has been used as an indicator of 5-HT synthesis (Boldrini et al., 2005). In this study, gerbils that received alcohol showed a depressive state confirmed by a decrease of 5-HT synthesis.

In the present results, cell proliferation in the dentate gyrus was decreased with increasing alcohol administration. Hippocampal neurogenesis is highly associated with learning, memory, and mood (Malberg et al., 2000; Morris et al., 2010). Proliferating hippocampal cells that can differentiate into neurons in the hippocampal dentate gyrus are commonly detected by BrdU assay. Previous studies showed that the rate of BrdU-positive neurons in the hippocampal dentate gyrus was decreased by psychiatric disorders including depression (Malberg and Duman, 2003), and that several antidepressant treatments, such as tricyclic antidepressants, serotonin reuptake inhibitors, and monoamine oxidase inhibitors, improved depression-like behaviors by activation of neurogenesis in the hippocampal dentate gyrus (Malberg et al., 2000; Santarelli et al., 2003; Warner-Schmidt and Duman, 2006). Herrera et al. (2003) reported that chronic alcohol intake induced depression-like symptoms with reduction of proliferating cells labeled with BrdU. Alcohol-induced depression was prevented by increasing proliferation of hippocampal neurons (Nixon, 2006; Morris et al., 2010). Based on previous studies, suppression of neurogenesis in the hippocampal dentate gyrus is one of the hallmarks of alcohol-induced depression. In this study, gerbils that received alcohol showed a depressive state confirmed by a decrease of cell proliferation.

In the present results, expression of BDNF, TrkB, p-ERK1/2, and Bcl-2 in the hippocampus was decreased with increasing alcohol administration. The expression of total ERK1/2 was not changed by alcohol administration in this study. The gerbils that received alcohol showed suppression of BDNF-ERK signaling molecules. Many studies have suggested that

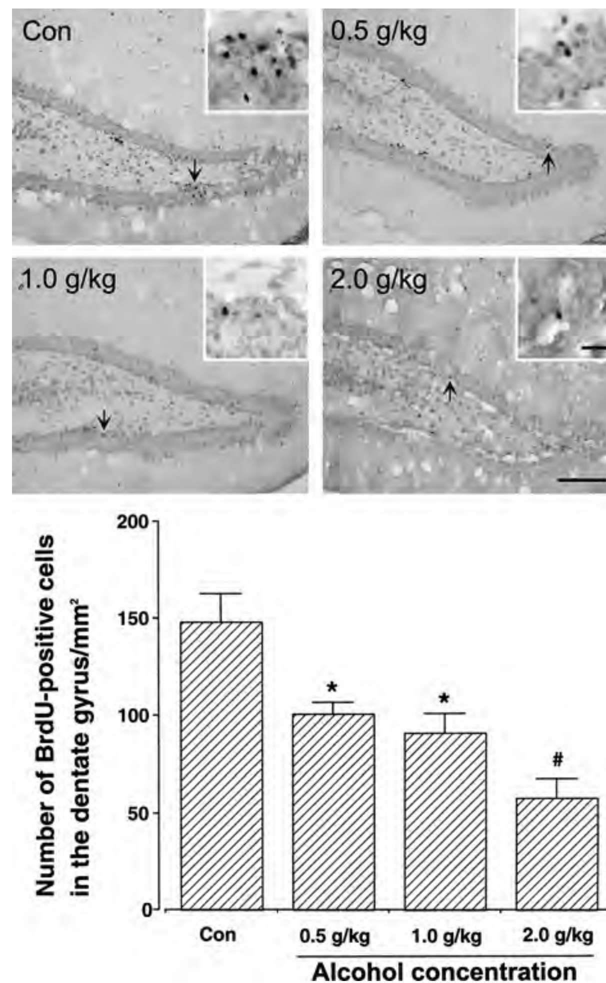

Figure 3. Effect of alcohol intake on cell proliferation in the hippocampal dentate gyrus. Upper panel: photomicrographs of 5-bromo-2′-deoxyuridine (BrdU)-positive cells in the dentate gyrus of hippocampus. The scale bar represents 800 μm. Inserted images are to enlarge arrow regions. The scale bar represents 200 μm. Lower panel: number of BrdU-positive cells in each group. * represents $P < 0.05$ compared to the control group. # represents $P < 0.05$ compared to the 0.5 g/kg alcohol-treated group. Con = control group.

hippocampal neurogenesis may be mediated by induction of BDNF (Angelucci et al., 2005; Stevenson et al., 2009). BDNF promotes the survival and function of neurons in the hippocampus (Moonat et al., 2010). Particularly, BDNF has a critical role in the treatment of depression, because administration of several antidepressants increased BDNF expression in the hippocampus and ameliorated FST results observed in depression (Altar, 1999; Angelucci et al., 2005).

Coyle and Duman (2003) reported that BDNF activated the ERK-MAPK pathway, which improved depressive symptoms. BDNF signaling, especially via MAPK, increases Bcl-2 expression in the hippocampus through phosphorylation of ERK1/2 (Powrozek and Miller, 2009). Bcl-2 promotes cell survival via reducing the release of calcium and cytochrome *c* and enhancing mitochondrial calcium uptake (Adams and Cory, 1998). The level of Bcl-2 in the hippocampus was decreased in depression; in contrast, antidepressants and lithium not only indirectly up-regulated expression of BDNF, MAPK, and Bcl-2 in the hippocampus, but also enhanced hippocampal cell survival (Manji and Duman, 2001; Réus et al., 2011). These mechanisms are in accordance with the present results that alcohol intake suppressed BDNF expression and resulted in the suppression of its downstream molecules, pERK1/2 and Bcl-2, in the hippocampus.

Here in this study, alcohol intake accompanied suppression of 5-HT synthesis and TPH expression in the dorsal raphe and a decrease of cell proliferation in the hippocampal dentate gyrus. These results indicate that alcohol intake induced a depressive state in the gerbils. Based on the present results, inhibition of the BDNF-ERK signaling pathway by alcohol can be ascribed as one of the underlying mechanisms of alcohol-induced depression.

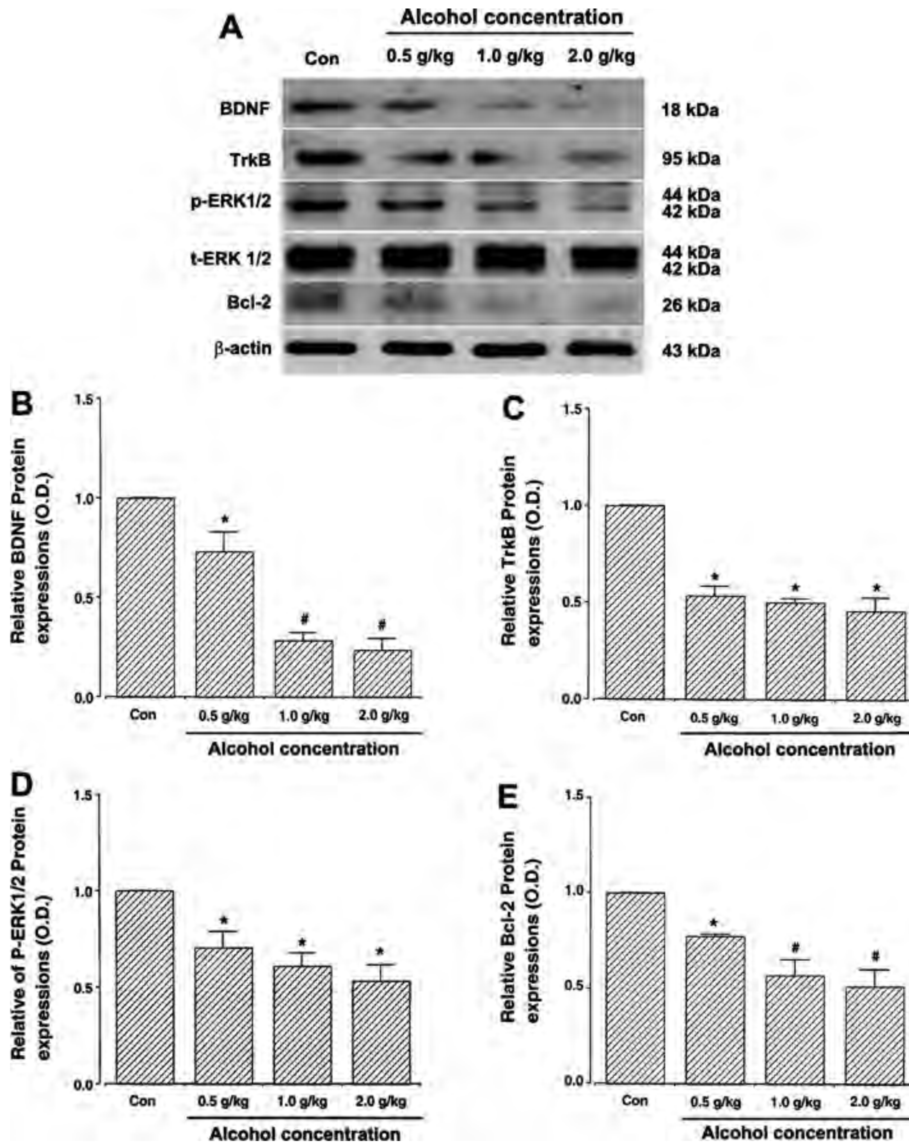

Figure 4. Effect of alcohol intake on brain-derived neurotrophic factor (BDNF)-extracellular signal-regulated kinase (ERK) signaling molecules. * represents $P < 0.05$ compared to the control group. # represents $P < 0.05$ compared to the 0.5 g/kg alcohol-treated group. Con = control group.

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