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Effect of Beta-Asarone on Impairment of Spatial Working Memory and Apoptosis in the Hippocampus of Rats Exposed to Chronic Corticosterone Administration

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Abstract

β-asarone (BAS) is an active component of *Acori graminei rhizoma*, a traditional medicine used clinically in treating dementia and chronic stress in Korea. However, the cognitive effects of BAS and its mechanism of action have remained elusive. The purpose of this study was to examine whether BAS improved spatial cognitive impairment induced in rats following chronic corticosterone (CORT) administration. CORT administration (40 mg/kg, i.p., 21 days) resulted in cognitive impairment in the avoidance conditioning test (AAT) and the Morris water maze (MWM) test that was reversed by BAS (200 mg/kg, i.p). Additionally, as assessed by immunohistochemistry and RT-PCR analysis, the administration of BAS significantly alleviated memory-associated decreases in the expression levels of brain-derived neurotrophic factor (BDNF) and cAMP-response element-binding protein (CREB) proteins and mRNAs in the hippocampus. Also, BAS administration significantly restored the expression of Bax and Bcl-2 mRNAs in the hippocampus. Thus, BAS may be an effective therapeutic for learning and memory disturbances, and its neuroprotective effect was mediated, in part, by normalizing the CORT response, resulting in regulation of BDNF and CREB functions and anti-apoptosis in rats.

Key Words: β-asarone, Memory, Corticosterone, Brain-derived neurotrophic factor, cAMP-response element-binding protein, Apoptosis

INTRODUCTION

Chronic stress results in the dysregulation of hypothalamic-pituitary-adrenal (HPA) axis in the neuroendocrine system, leading to secretion of a stress hormone, glucocorticoids (GCs) (Walesiuk and Braszko, 2010; Kim *et al*., 2014). Chronic exposure to GCs may results in impaired spatial cognition and neuroendocrine and plasticity abnormalities, but it is also considered a risk factor for pathologies such as Alzheimer's disease (AD)(Dobarro *et al*., 2013). It has also been showed that initially higher serum GC levels in the pre-dementia clinical stage of AD predict a more rapid cognitive decline (Csernansky *et al*., 2006; Lee *et al*., 2014). Therefore, elevation of circulating GC concentrations by systemic administration of corticosterone (CORT) or GC receptor agonists exerts an inhibitory influence on cognition and memory function in rodents and humans (Aisen *et al*., 2000; Roosendaal *et al*., 2004). It has been suggested that the effects of GCs on memory depend on noradrenergic activation of the amygdala and dysregulation of HPA axis of the hippocampus (Roozendaal *et al*., 2006; Dobarro *et al*., 2013). Thus, hippocampal neurons, which are known to play an important role in learning and memory, are particularly vulnerable to neuronal injury induced by chronic administration of CORT (Plaschke *et al*., 2006), resulting in deficits in spatial memory and synaptic plasticity (Walesuik *et al*., 2006; Sajadi *et al*., 2007).

In addition, new findings concerning the activation of hippocampal brain-derived neurotrophic factor (BDNF) and

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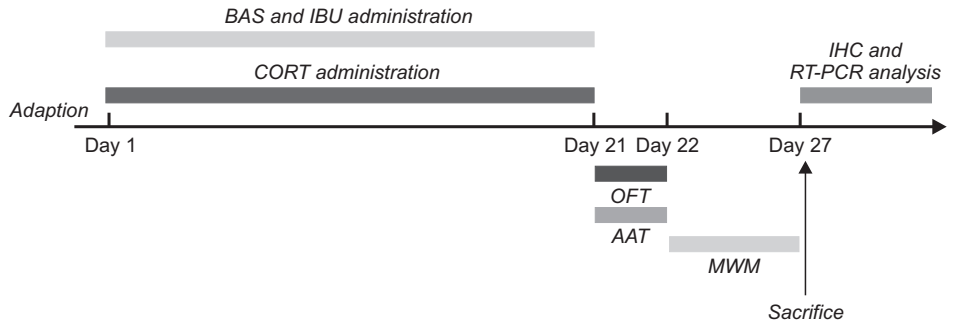

Fig. 1. Experimental schedules for CORT administration to induce memory impairments in rats. The experiment was designed to explore the efficacy of BAS in healing chronic CORT-induced memory impairment in an animal model using behavioral and neurobiological methodologies.

cAMP-response element-binding protein (CREB) in the brain as novel therapeutics for healing memory deficits have been reported through several basic and clinical studies (Vaynman *et al*., 2008; Willians *et al*., 2008). Several studies have demonstrated that the CREB-BDNF pathway is important in long-term memory formation (Bekinschtein *et al*., 2008; Saura and Valero, 2011) and that CORT-induced memory deficits lead to a significant reduction in BDNF and CREB expression in the hippocampus as well as poor performance in learning and memory tests (Lee *et al*., 2014).

Also, neuronal apoptosis in the hippocampus disturbs learning ability and memory function, and excessive neuronal apoptosis in the hippocampus contributes to memory dysfunction (Kuhn *et al*., 2005; Sun *et al*., 2009). In particular, stress in through to induced neuronal death, possibly through apoptosis (Lucassen *et al*., 2001; Kudryashov *et al*., 2002). As mentioned above, hippocampal BDNF and CREB function and apoptosis play important roles in pathological conditions of the central nervous system (CNS) and are associated with neurodegenerative diseases such as AD and Parkinson's disease (Alkadhi *et al*., 2010).

An extract of *Acori graminei rhizoma* (AGR), an indigenous medicinal plant is used traditionally as an ingredient of various cocktail preparations, such as Kaixinsan and Chong-Myung-Tang, which have been used for the treatment of morbid forgetfulness in Korea, China, and other Asian countries (Chen *et al*., 2014). In our and other experimental studies, cocktail preparations including AGR have been used clinically as traditional medicines against stroke, vascular dementia, and AD by native doctors (Wang *et al*., 2010; Lee *et al*., 2012). β-Asarone (BAS, *cis*-2,4,5-trimethoxy-1-propenylbenzene), a small molecule, was found to be an essential component of AGR, and to influence learning and memory (Lee *et al*., 2004; Lee *et al*., 2010). Moreover, recent studies on BAS have reported that it improves multiple physiological actions, produces a variety of pharmacological actions in the CNS, and has nootropic and neuroprotective effects *in vitro* and *in vivo* (Cho *et al*., 2002; Zou *et al*., 2011). It has been reported that BAS can attenuate neuronal apoptosis in the rat hippocampus and may be a potential candidate for development as a therapeutic agent to manage cognitive impairment associated with conditions such as AD (Li *et al*., 2010; Liu *et al*., 2010). Several studies have shown that the administration of BAS can reduce the toxicity of excitatory amino acids in the epileptic rat brain and increase the expression of c-fos (Liu *et al*., 2010). Additionally, BAS can reduce the injuries to blood vessel endothelium and nerve cells of the cortex, and improve cognitive function in β-amyloid hippocampus injection rats (Geng *et al*., 2010; Li *et al*., 2012). In addition, α-asarone protected neurons against β-amyloid (25-35)-induced neurotoxicity and spatial memory impairment by inhibiting the effects of nitric oxide (NO) overproduction in the hippocampus and temporal cortex (Limón *et al*., 2009). But so far the mechanisms on the deficits in spatial learning abilities of the constituents of BAS were not yet fully explored. BAS readily passes through the blood brain barrier, and appears to be distributed widely without obvious target regions in the brain (Li *et al*., 2010; Liu *et al*., 2010).

The aim of the present study was to evaluate the ability of BAS to improve learning and memory in rats exposed to repeated CORT-induced spatial memory impairments as measured by their performance on the using an avoidance conditioning test (AAT) and the Morris water maze (MWM) test. The manner in which these activities were related to the expression of BDNF and CREB and apoptosis in the hippocampus was also investigated.

MATERIALS AND METHODS

Animals

The present study utilized adult male Sprague-Dawley (SD) rats weighing 200-220 g (6 weeks-old, Samtako Animal Company; Seoul, Korea). All rats were housed in a limited access rodent facility with five rats per polycarbonate cage. The room controls were set to maintain the temperature at 22 ± 2°C and the relative humidity at 55 ± 15%, the cages were lit by artificial light for 12 h each day, and sterilized drinking water and a standard chow diet were supplied ad libitum throughout the experiments. All efforts were made to minimize the number and suffering of animals.

CORT and BAS administration

Different groups of rats, 6-7 animals per group, were used for drugs treatment and tests. All the experimental animals including control and drug-treatment groups were administration. The standard doses of BAS in the rat and considering the long-term treatment used in the present study was based on previous study (Li *et al*., 2012). BAS (50, 100 and 200 mg/kg, body weight; Sigma-Aldrich Chemical Co. St. Louise, MO, USA) and the positive drug ibuprofen (40 mg/kg, IBU; Sigma-

Aldrich Chemical Co.) were administered by intraperitoneally (i.p.) in a volume of 1 ml/kg 30 min prior to the CORT (40 mg/kg, Sigma-Aldrich Chemical Co.) injection for 21 days. BAS and IBU were dissolved in 0.9% physiological saline before use. CORT, which was dissolved in a saline solution containing 0.1% dimethyl sulfoxide and 0.1% Tween-80, was administrated by subcutaneously (s.c) in a volume of 5 ml/kg once daily for 21 days (Lee *et al*., 2014). As a vehicle control, animals in the control (CON) group were subcutaneously given the equivalent volumes in saline to the final concentration of 10% ethanol in a volume of 10 ml/kg. The CON group and CORT group also received saline instead of BAS as a vehicle control in an equal volume for a period of 21 days. This CORT dose was selected because it induces plasma levels of the steroid comparable to those elicited by substantial stress (Lee *et al*., 2014). The CORT and vehicle injections were given in the morning between 9 and 10 am once daily for 21 consecutive days. All drugs were freshly prepared right before every experiment. The entire experimental schedules of all drug administration and behavioral examinations are shown in Fig. 1. The following parameters were measured to monitor the effects of stress-induced physiological alteration by repeated CORT injection: changes of serum CORT levels. Behavioral testing for cognitive impairments was done 24 h after the end of drug injection. To avoid carryover from one test to another, each behavioral test was performed on separate or interval between each behavioral test. Following behavioral testing, all rats were sacrificed and their brain tissues were collected immediately for use in the study or stored at -70°C for later use.

Corticosterone measurement

For this, the unanesthetized rats were rapidly decapitated, and blood was quickly collected via the abdominal aorta. Rats were randomly divided into six groups consisting of four individuals each, the same as above. The blood samples were centrifuged at 4,000 *g* for 10 min, and serum was collected. The CORT concentration was measured by a competitive enzyme-linked immunosorbent assay (ELISA) using a rabbit polyclonal CORT antibody (Novus Biologicals Corticosterone kit; Novus Biologicals, LLC, Littleton, CO, USA) according to the manufacturer's protocol. Samples (or standard) and conjugate were added to each well, and the plate was incubated for 1 h at room temperature without blocking. After the wells were washed several times with buffers and proper color developed, the optical density was measured at 450 nm using an ELISA reader (MultiRead 400; Authos Co., Vienna, Austria).

Active avoidance conditioning test

The AAT test measures the ability of an animal to avoid an aversive event and provides a way to assess associative learning and memory (Grauer *et al*., 2009). Active avoidance conditioning was performed as previously described (Grauer *et al*., 2009) before starting the MWM assay. The Gemini Avoidance System (SD Instruments) was used for this experiment. Basically, each rat was individually placed in a two-way shuttle box (23 cm×50 cm×23 cm) composed of two stainless steel rods (3 mm diameter, 1 cm apart) and two 28 V DC lights. Electric shocks were transmitted to the gird floor by an isolated shock generator (Behbood Pardaz Co. Iran). First, after a 5 min period of habituation in the shuttle box, the rats were subjected to 50 avoidance trails on variable interval schedule (range=7.5-22.5 s). Each trial consisted of the warning tone and the stimulus light (conditioning stimulus) for 10 s. If an animal crossed through the archway during the initial 10 s of each trial, the tone and the light were terminated. A conditioned avoidance response was defined as crossing to the opposite chamber within the initial 10 s of each trial. Exactly 24 h after the conditioned avoidance response, rats were again place in the shuttle box. Each trial consisted of the warning tone and the electrical shock (0.5 mA; unconditioning stimulus) for 10 s, presented through the grid floor on the side where the rats were located. If an animal crossed through the archway after the electrical shock was initiated, the tone and the shock were terminated. An escape response was defined as crossing to the opposite chamber within the initial 10 s of each trail. The retention test (escape response) for memory was performed to 20 avoidance trails. If no escape response to the shock occurred within 10 s, the shock and tone-conditioning stimulus were discontinued and an escape failure was recorded.

Morris water maze test

After the AAT test, the MWM test was performed in a small circular pool (2.0 m in diameter and 0.35 m deep) made of polypropylene and internally painted white. The pool was half-filled with water to 30 centimeters in depth. The water in the pool was made opaque by adding 1 kg skim milk powder and continuously maintained at 22 ± 2°C. The pool was divided into four quadrants of equal area. During the MWM test, an escape platform (15 cm in diameter) was located in one of four sections of the pool being hidden 1.5 cm below the water surface and apart approximately 50 cm from the sidewalls. Several visual cues were placed around the pool in plain sight of the animals. A digital camera was mounted to the ceiling straight above the center of the pool and was connected to a computerized recording system equipped with a tracking program (S-MART: PanLab Co., Barcelona, Spain), which permitted on- and off-line automated tracking of the paths taken by the rats. In the hidden platform trial for acquisition test, the MWM test was initiated on the 23th day after the BAS and CORT administration commenced. The animals received three trials per day. The rats were trained to find the hidden platform, which remained in a fixed location throughout the test. The trials lasted for a maximum of 180 s, and the escape latency was expressed by the swimming time to find the submerged platform in the pool. The animals were tested three trials per day for 5 days, and they received a 60-s probe trial on the sixth day. Finding the platform was defined as staying on it for at least 4 s before the acquisition time of 180 s ended. When the rat failed to find the platform in the limited time in first trial of hidden platform test, the rats should be placed on the platform for 20 s and assigned a latency of 180 s. Between one trail and the next, the water in the pool was stirred to remove olfactory traces of previous swim patterns. The entire schedule proceeded for six days and each animal had three trials for training per day with 30 to 40 min inter-trial interval. In the probe trial for retention test, a rat was placed in the quadrant located diagonally from the target quadrant and allowed to swim to the quadrant from which the escape platform had been removed for a maximum of 60 s. The probe trial was expressed by the ratio of the time spent and the distance for searching the platform in the target quadrant to total duration spent for swimming in the pool.

Open field test

Before the AAT and MWM tests, several parameters, such as defecation and time in a center within the first 5 min, likely gauge some aspects of emotionality including anxiety (Lee *et al*., 2014). Animals were treated in an open field apparatus in a room. All rats were placed individually in a rectangular container made of dark polyethylene (60×60×30 cm), which provided the best contrast for the white rats in a dimly lit room, and all locomotor activities (animal's movements) was measured by a video camera mounted above the center of the maze. The speed and distance of the movements of the subject were monitored by a computerized video-tracking system using the S-MART program (PanLab Co., Barcelona, Spain). Following a habituation period of 5 min, the total distance traveled in the container (measured in cm) was recorded. The number of rearing events of the rats was also recorded to analyze locomotor activity in the open field test. The floor surface of each chamber was cleaned thoroughly with 70% ethanol between tests for different subjects.

Immunohistochemistry

To conduct the immunohistochemical analyses, three rats from each group were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused through the ascending aorta with 0.9% saline followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brains were removed in a randomized order, post-fixed overnight, and cryoprotected with 20% sucrose in 0.1 M PBS at 4°C. Coronal sections (30 μm) were cut through the hippocampus using a cryostat (Leica CM1850, Leica Microsystems Ltd.; Nussloch, Germany) according to the rat atlas of Paxinos and Watson (Paxinos and Watson, 1986). The sections obtained from the rats were immunostained for BDNF and CREB expression using the avidin-biotin-peroxidase complex (ABC) method. Briefly, the sections were incubated with primary rabbit anti-BDNF antibody (1:200 dilution, Cell signaling, Boston, MA, USA) and primary rabbit anti-CREB antibody (1:200 dilution, Cell signaling, Boston, MA, USA) in PBS plus 0.3% Triton X-100 (PBST) for 72 h at 4°C. Next, the sections were incubated for 120 min at room temperature with secondary antibodies (1:200 dilution, Vector Laboratories Co.; Burlingame, CA, USA) in PBST containing 2% normal serum. To visualize immunoreactivity, the sections were incubated for 90 min in ABC reagent (Vectastain Elite ABC kit, Vector Labs. Co.) and then in a solution containing 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) and 0.01% H_2O_2 for 1 min. Finally, the tissues were washed in PBS, briefly rinsed in distilled water, and mounted individually onto slides.

Images were captured using the AxioVision 3.0 imaging system (Carl Zeiss, Inc.; Oberkochen, Germany) and processed using Adobe Photoshop (Adobe Systems, Inc.; San Jose, CA, USA). The numbers of BDNF- and CREB-labeled cells in the stained sections of the hippocampus were quantified at a magnification of 200× by an observer blinded to the experimental groups. The immunopositive cells were localized anatomically within at least three different hippocampus sections per rat brain that were randomly chosen from equivalent levels of serial sections along the rostral-caudal axis according to the stereotactic rats brain atlas of Paxinos and Watson (Paxinos and Watson, 1986). Only cells in which the staining reached a predefined value above background were considered to be immunopositive; distinct brown spots indicating BDNF- and CREB-immunopositive cells were observed in the hippocampus. Differences in brightness and contrast among the raw images were not adjusted, to exclude any possibility of the subjective selection of immunoreactivity.

Total RNA preparation and RT-PCR analysis

The hippocampus from four rats in each group was isolated. After decapitation, the brain was quickly removed and stored at -80°C until use. Total RNA was isolated from the brain samples using TRIzol® reagent (Invitrogen Co., Carlsbad, CA, USA) and was used to extract RNA according to the supplier's instruction. Complementary DNA was synthesized from total RNA with reverse transcriptase (Takara Co., Shiga, Japan). BDNF, CREB, Bax and Bcl-2 mRNAs expression levels were determined by the reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed using a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA, USA). All primers were designed using published mRNA sequences and primer design software (Primer 3; The Whitehead Institute for Biomedical Research, Cambridge, MA, USA; www.genome.wi.mit.edu), offered through the web site. The PCR products were separated on 1.2% agarose gels and stained with ethidium bromide, and the density of each band was analyzed using an image-analyzing system (i-Max™, CoreBio System Co., Seoul, Korea). Complementary DNA expression levels were determined by calculating the relative density of each BDNF, CREB, Bax and Bcl-2 mRNAs band to GAPDH.

Statistical analysis

All measurements were performed by an independent investigator blinded to the experimental conditions. Results in figures are expressed as mean ± standard error of means (SE). Differences within or between normally distributed data were analyzed using an analysis of variance (ANOVA) using SPSS (Version 13.0; SPSS, Inc., Chicago, IL, USA) and a Tukey's *post-hoc* test. A p value of <0.05 was considered to indicate statistical significance.

For statistical analysis of water maze data, the effect of training on the acquisition of the water escape task was assessed by using one-way ANOVA with the repeated measure factor sessions (number of days) followed by appropriate Tukey's *post hoc* analysis. For probe trial in the water maze, within-group differences in the time and distance spent in each quadrant, and AAT, OFT, immunohistochemical data and PCR analysis were also analyzed by a one-way ANOVA followed by Tukey's *post hoc* test.

RESULTS

Effect of BAS on repeated CORT-induced changes of serum CORT levels

Acute CORT injection induces a large increase in serum CORT level, which gradually increased as CORT injection was repeatedly applied to the rats, probably due to adrenal habituation (Lee *et al*., 2010; Wüppen *et al*., 2010). The ELISA analysis demonstrated that repeated CORT injection significantly increased the serum CORT concentration in the rats by 281.57% ($p<0.05$), as compared to CON group (Fig. 2). It indicated that the repeated CORT injection was sufficiently stressful despite the evoked CORT response to repeated CORT

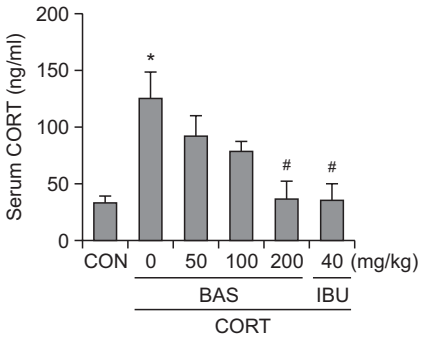

Fig. 2. Effects of BAS administration on the serum levels of CORT in rats during chronic CORT injection. *p<0.05 vs. the CON group, #p<0.05 vs. the CORT group.

injection was significantly less than the response to single CORT injection (data not shown). In these results, exogenous CORT-induced memory impairments was exploited to develop a chronic stress model in the rats. However, daily administration of BAS significantly inhibited the repeated CORT-induced increase in serum CORT level as compared to CORT group (p<0.05).

Effect of BAS on repeated CORT-induced memory impairment

To determine whether BAS promotes the recovery of memory dysfunction, BAS was administration to the rats with CORT-induced impairment of memory, and their memory and cognitive functions were examined by the AAT (Fig. 3). The rats in the CORT group showed significant decreases of latencies to enter the opposite chamber for conditioned avoidance responses, as compared to those in the CON group (p<0.01; Fig. 3A). 100 mg/kg or 200 mg/kg CORT-induced rats showed slightly increase of reductions in latencies to enter the opposite chamber for conditioned avoidance responses (CAR), as compared to those in the CORT group, although the findings were only minimally significance. After CAR trials for 24 h, the effect of BAS administration on retention latency, indicated by the latencies times for entering the opposite chamber, was examined by applying electric shock to gird floor of the shuttle box in the AAT. In the retention, the rats in the CORT group exhibited significant increases of latencies to enter the opposite chamber for escape failures, as compared to those in the CON group (p<0.01; Fig. 3B). However, in the CORT+BAS200 group, they showed decreased latencies to enter the opposite chamber for escape failures (EF), as compared to those in the CORT group (p<0.05). This study indicated that repeated exposures to CORT severely impaired short-term memory in the rats and that BAS treatment significantly attenuated CORT-induced memory deficit. The rats in the CORT group showed a reduction of the acquisition of CAR and an increase in EF. Moreover, the percentage of CAR or EF observed in the CORT+BAS200 group was similar to that in the CON group. It also indicated that the recovery of cognitive functioning on stress-related memory impairment in the CORT+BAS200 group was almost compatible with that in the CORT+IBU group.

The effect of BAS treatment on swimming to reach the submerged platform in the MWM test is elucidated in Fig. 3C, 3D. Rats in the CON group rapidly learned the location of the submerged hidden platform and reached it within 25 sec on day 5 of the trials. The CORT group showed marked retardation in escape latency, probably due to memory deficits resulting from CORT-induced impairment of learning and memory. Analysis of escape latency revealed that rats in the CORT+BAS200 group had significantly reduced swimming latency as compared with those in the CORT group (p<0.05 on day 3 and 5; Fig. 3C). To investigate the effect on spatial memory, the performance in the probe trial on day 6 was examined by analyzing the percentages of time and distance spent swimming to the expected position of the platform (Fig. 3D, 3E). The swimming times and distances were reduced in the rats that swam directly and without confusion to the target area where the platform had been located. The rats with CORT injection showed severe impairment of spatial performance in the MWM test (p<0.01). The rats in the 200 mg/kg BAS-treated group spent more time around the platform area than did those in the CORT group (p<0.05). Based on these results, rats treated with 200 mg/kg BAS were suggested to show greater improvement in acquisition during the hidden platform trial and, accordingly, reached the platform quicker than the CORT-treated rats. The results also indicated that the swimming latency of the CORT-induced rats receiving 200 mg/kg BAS was similar to that of rats receiving 40 mg/kg IBU. The CORT group was not significantly different from the other groups in terms of the mean swimming speed, as calculated by dividing the total swimming distance by the latency (Fig. 3F).

In addition to MWM test, an open field test (OFT) in the present study was used to evaluate spontaneous locomotor activity and exploratory behavior among the rats receiving a CORT injection for 21 days (Fig. 3G). These results indicated that the rats in all groups had no their locomotor activities (motor function) and total numbers of rearing (exploration activities) in the OFT. Because any significant differences of locomotor activities and total numbers of rearing were not observed between all groups in the OFT, it could be suggested that observed impairment of memory of the rats with repeated CORT injection were not attributed to the differences of their locomotion activities. It is may reflect an active responses and water avoidance stress when the animal is confronted with a MWM test. However, our results suggest that rats in all groups displayed no anxiolytic-like behaviors in the OFT after a pretest stress exposure in the MWM test. This indicates that administration of BAS did not affect active responses or psychomotor function as measured by the rats' performance in the MWM test.

Effects of BAS on CORT-induced changes of BDNF and CREB in the hippocampus

After the behavioral measures were completed, BDNF- or CREB-like immunoreactivity were analyzed in the hippocampal neuronal areas. Compared with the CON group, BDNF- and CREB-like immunoreactive cells in the hippocampus of the CORT group decreased to 61.14% and 61.82%, which indicates that repeated CORT injection results in a significant decrease in BDNF and CREB expression, respectively (p<0.05; Fig. 4A). Compared with the CORT group, there was a significant increase in the number of BDNF-immunoreactive neuronal cells in the hippocampus of the CORT group (p<0.05; Fig. 4B). However, compared with the CORT group, there was a slight increase in the number of CREB-immu-

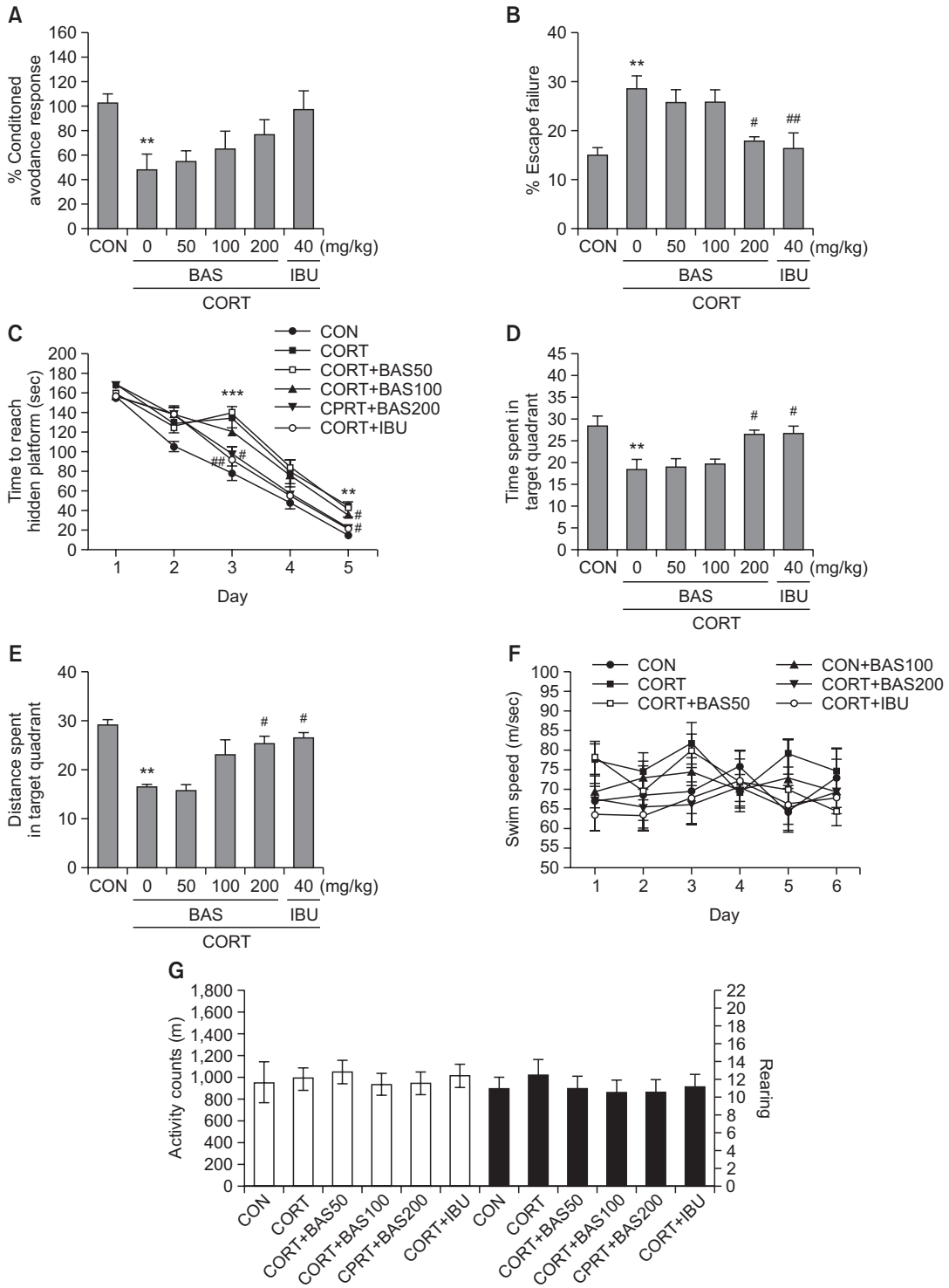

Fig. 3. Effects of BAS on the latency to enter the opposite chamber for conditioned avoidance responses (A) and escape failures (B) in the active avoidance-conditioning test, and time to escape (latency) from water during acquisition trials using a submerged platform (C), the percentages of time spent in target quadrant (D), the percentages of distance spent in target quadrant (E) and swimming speed (F) in the Morris water maze test. Effects of BAS administration on activity counts of locomotor activity and total number of rearing in the open field test during chronic CORT injection (G). $^{**}p<0.01$, $^{***}p<0.001$ vs. the CON group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. the CORT group.

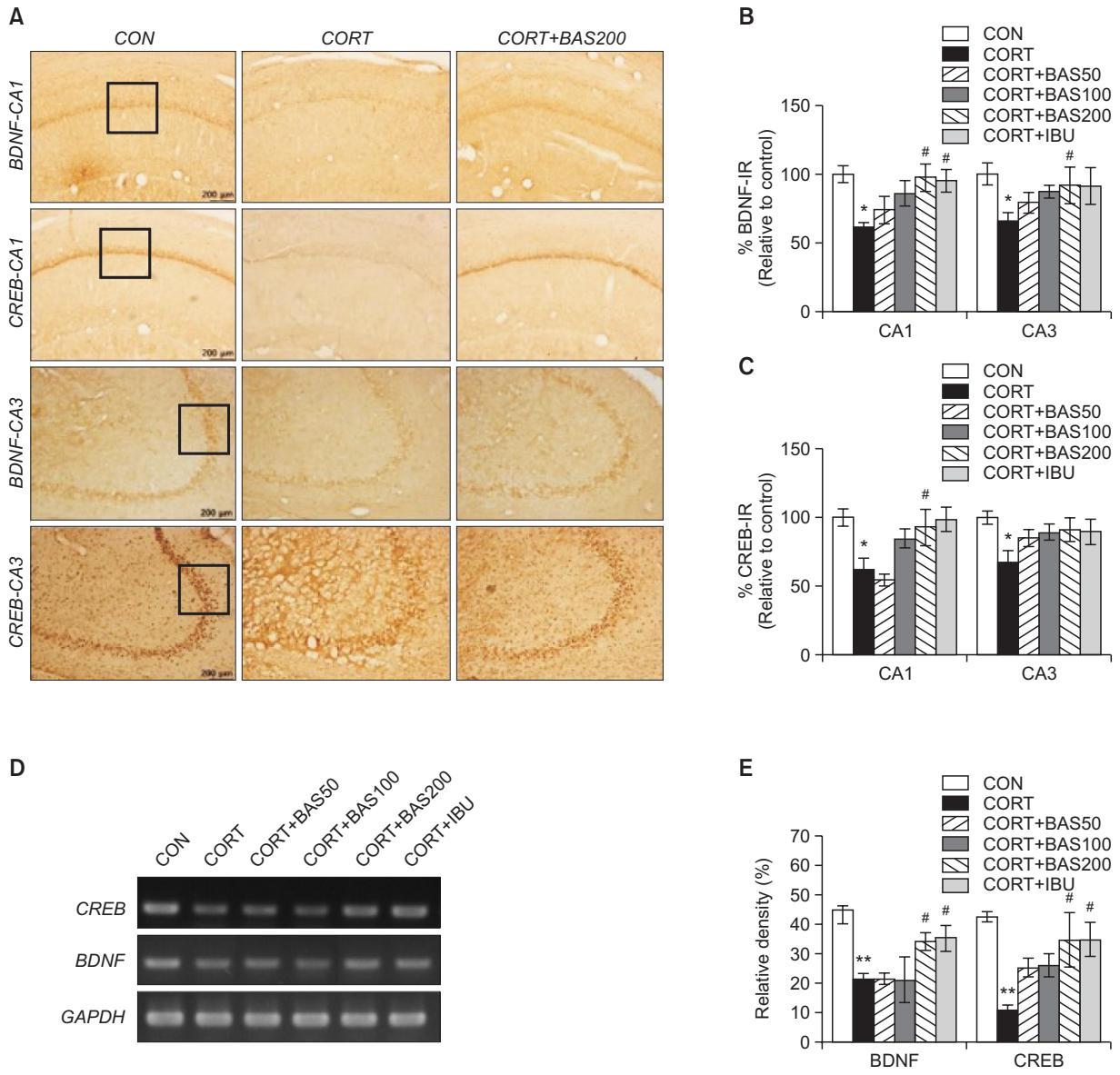

Fig. 4. Effects of BAS administration on the mean number of brain-derived neurotrophic factor (BDNF) and cAMP-response element-binding protein (CREB)-stained hippocampal areas and mRNAs after the Morris water maze test. Representative photographs and the relative percentage values are indicated in (A) and (B and C), respectively. Scale bar represents 100 μm. PCR bands on agarose gel and their relative intensities are indicated in (D) and (E), respectively. The expression levels of BDNF and CREB mRNAs were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. *p<0.05, **p<0.01 vs. the CON group, $^{\#}p$<0.05 vs. the CORT group.

noreactive neuronal cells in the hippocampus region of the CORT+BAS200 group, although this result was only minimally significant group (Fig. 4C). The results also indicated that the number of BDNF-reactive neuronal cells in the hippocampus in rats receiving 200 mg/kg BAS was similar to that in rats receiving 40 mg/kg IBU.

The effects of BAS administration on the expression of BDNF and CREB mRNAs in the hippocampus of rats exposed to repeated CORT injection were investigated using RT-PCR (Fig. 4D). There was a significant decrease in the hippocampal BDNF and CREB mRNA expression in the CORT group compared with the CON group (p<0.01; Fig. 4E). The decreased expression of BDNF and CREB mRNA in the CORT group was significantly restored to levels similar to those in the CON group by 200 mg/kg BAS (p<0.05). This indicates that the expression of BDNF and CREB mRNAs in the hippocampus of rats receiving 200 mg/kg BAS was similar to that of rats receiving 40 mg/kg IBU. We also demonstrated significant decreases in BDNF and CREB mRNAs expression in the rat hippocampus tissues following CORT-induced memory deficits and showed that administration of BAS restored BDNF and CREB mRNAs expression levels.

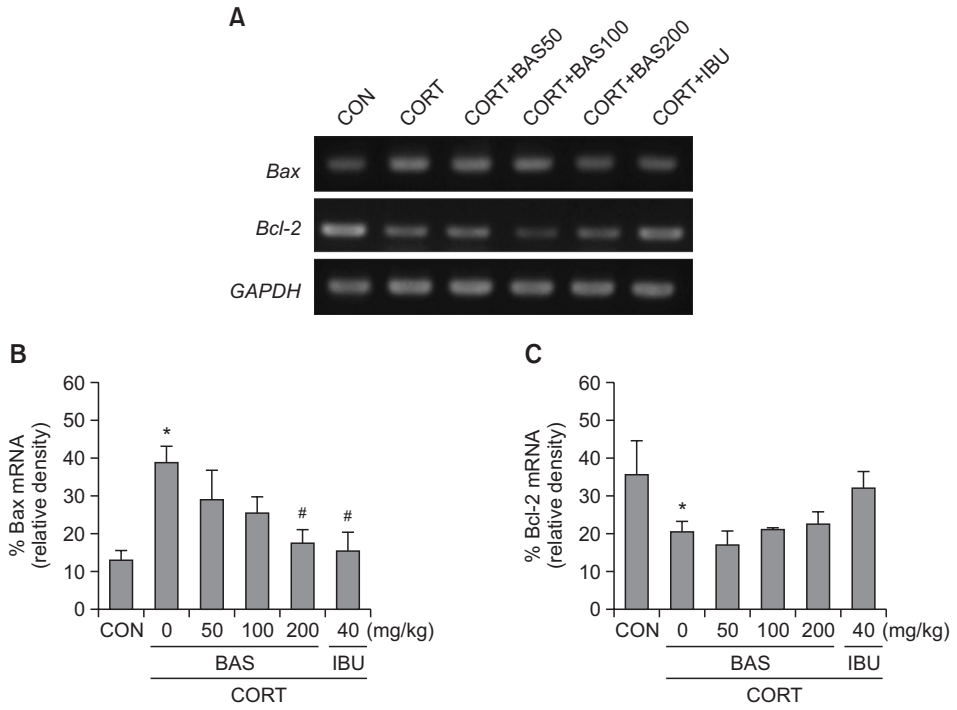

Fig. 5. Effects of BAS administration on the expression of Bax and Bcl-2 mRNA in rats with CORT-induced hippocampal impairment. *p<0.05 vs. the CON group, $^{\#}p$<0.05 vs. the CORT group.

Effects of BAS on CORT-induced expression of Bax and Bcl-2 mRNAs in the hippocampus

The effects of BAS administration on the expression of Bax and Bcl-2 mRNAs in the hippocampus of rats exposed to repeated CORT injection were investigated using RT-PCR (Fig. 5). There were a significant increased expression of Bax mRNA and decreased expression of Bcl-2 mRNA in the CORT group compared with the CON group (p<0.05 and p<0.01). The increased expression of Bax mRNA in the CORT group was significantly restored to levels similar to those in the CON group by 200 mg/kg BSA (p<0.05). This indicates that the expression of Bax mRNA in the hippocampus of rats receiving 200 mg/kg BAS was similar to that of rats receiving 40 mg/kg IBU.

DISCUSSION

The present study demonstrates that in an animal model, treatment with BAS can reverse impaired cognition induced by chronic stress. In addition to the maintenance of cognitive function, normalizing of blood serum CORT levels, and reversal of BDNF and CREB expression in the hippocampus were also seen in animals treated with BAS. Also, administration of BAS produced decreased Bax mRNA expression in the hippocampus associated with repeated CORT-induced memory impaired in male rats. Memory impairments induced by repeated injections of CORT were exploited to develop a chronic stress model in rats (Nacher *et al*., 2004; Plaschke *et al*., 2006). In this animal model, the forced maintenance of high CORT levels can affect cognition by reducing memory capacity, and this may be correlated with the progression or exacerbation of chronic stress in humans (Walesiuk and Braszko, 2006; Walesiuk *et al*., 2010). BAS restored serum CORT levels to near normal levels towards the end of the 2-week treatment period, suggesting that BAS inhibited stress-related HPA axis dysfunction and related behavior disorders and BDNF and CREB expression via hippocampal mechanisms. These findings may help explain the manner in which the administration of BAS affects the hippocampus and the biochemical and behavioral signals induced by reduced CORT levels in serum.

Stress is an unavoidable life experience that can disturb cognitive processes and neuroplasticity (Rashidy-Pour *et al*., 2009). In the present study, rats were subjected to chronic administration of CORT, which may emulate that psychosocial or physiological stress that humans encounter in daily life, for 21 consecutive days and then treated with vehicle or BAS.

To identify the effects of BAS on two different types of memory, cognitive memory and spatial learning, the AAT and MWM, respectively were used. In the active avoidance paradigm, rats learn to avoid an aversive stimulus by initiating a specific locomotor response. During AAT, rats are placed in a two-compartment shuttle box and have to learn the association between a conditioned stimulus (tone or light) and an unconditioned stimulus (footshock) (Janak *et al*., 1994). In present study, it showed that CORT significantly shortened acquisition of a one-way step-through active avoidance response and impairs retention of active avoidance conditioning in rats. The administration of BAS significantly decreased the escape response and improved the ability of the animals to learn to active learning in the memory retention trial following the chronic administration of CORT. Also, to assess spatial learning and memory in rats, the MWM is more advantageous than other conventional mazes such as the T maze and the

radial-arm maze. The MWM is a hippocampus-dependent memory task, frequently used for demonstrating cognitive deficits and to examine permanent spatial learning capability and reference memory in rodents (Janasson, 2005). During the trial sessions in the MWM, the chronically stressed rats had significantly longer that normal escape latencies to reach the platform and showed a deficit in spatial learning. In the chronically stressed group that received BAS the animals learned faster and had shorter escape latencies than the chronic stress group. Moreover, the chronically stressed rats without BAS treatment performed poorly in subsequent testing compared with non-stressed rats on probe trials 24 h after acquisition, indicating impaired memory recall and retrieval. BAS prevented these behavioral abnormalities and restored both memory measures (spatial memory and learning) in chronically stressed rats. A similar effect was seen after chronic treatment with the drug ibuprofen (Browne *et al*., 2006). Thus, the present results support and confirm our hypothesis that BAS could ameliorate spatial learning and memory dysfunction induced by chronic stress.

An open-field test was also performed to rule out any confounding motor impairment that might influence outcomes in many behavioral tests of depression. No significant individual difference in locomotor activity was observed between groups, suggesting that the administration of BAS did not affect sensorimotor performance. Accordingly, the changes in behavioral performance in the MWM task were likely due to improved memory, not differences in sensorimotor function, motor output, or limb flexibility.

The hippocampal formation has one of the highest densities of GC receptors in the mammalian brain and participates in GC-mediated negative feedback of the HPA axis (Xu *et al*., 2009). In the rat hippocampus CORT has been shown to regulate neuronal metabolism, physiological function, gene expression, and to alter cell morphology (Sajadi *et al*., 2007). The involvement of the hippocampus in memory formation and in stress may be crucial for the cognitive deficits that commonly occur in an individual subjected to chronic stress (Roozendaal *et al*., 2006). Consistent with well-known views, structural integrity of the hippocampus is necessary for certain types of learning and memory, such as declarative and spatial memories (Xu *et al*., 2009).

To identify other BAS-related mechanisms of memory improvement, the effects of BAS on BDNF and CREB expression in the hippocampus, believed to be key molecules in the formation of memory, were investigated. Chronic CORT-induced memory deficits induced significant reductions in BDNF and CREB mRNA expression in the hippocampus and resulted in poor performance on hippocampus-dependent tasks (Bekinschtein *et al*., 2008; Saura, 2012).

It was found that the administration of BAS significantly prevented the reductions in BDNF and CREB mRNA expression induced by the chronic administration of CORT. These findings suggest that the beneficial effects of BAS are mediated by an increase in BDNF expression via the CREB signaling pathway and may be related to an increase in neuronal function and performance in memory tasks. The current results also suggest a correlation between protein and gene function in the reduced expression of BDNF and CREB in the hippocampus. CREB is a transcription factor important to the neuronal plasticity and long-term memory formation in the brain (Morris and Gold, 2012). CREB has been shown to be integral in the formation of spatial memory (Efimova *et al*., 2007). Also, the CREB active forms, phosphor-CREB are used as molecular markers in studies of the cellular substrates of learning and memory (Morris and Gold, 2012). The present study provided evidence that activation of transcription factors CREB can be used as molecular marker for mapping the processes of spatial memory in the nervous system. Our data provide further support for the hypothesis that the preventing neuronal impairment effect of BAS is at least in part correlated with the CREB or phosphor-CREB signaling pathway.

The elevated GCs levels following stress exert deleterious changes on hippocampal excitability, long-term potentiation, cerebral blood flow, and spatial learning memory (Endo *et al*., 1999; Kim *et al*., 2013). Chronic CORT injection causes neuronal death through apoptosis (Reagan and McEwen, 1997; Sapolsky, 1996). The Bcl-2 gene family plays important roles in apoptosis-regulating genes (Cory and Adams, 2002). Of these genes, Bax promotes cell death, whereas Bcl-2 inhibits apoptosis and promotes cell survival (Kim *et al*., 2010). Thus, we investigated the Bax and Bcl-2 mRNAs expression in the hippocampus after exposure to repeated CORT injection. We suggest that the administration of BAS significantly prevented the increase in Bax mRNAs expression induced by repeated CORT injection that resulted in memory deficits.

In summary, the present study demonstrates that, in a rat model of progressive memory deficits in neurodegenerative disease, exogenous CORT induced impaired neuronal function as well as associated memory and cognitive deficits. This was evidenced by performance on the AAT and MWM tests, and by protein and gene expression analyses. Administration of BAS significantly attenuated the symptoms of exogenous CORT-induced destruction, as indicated by improved cognitive functioning during behavioral tests, improved BDNF and CREB expression and apoptosis as well as exerted normalization of HPA axis. Thus, BAS may be a useful agent in preventing neuronal impairment such as that observed in the progression of memory deficits associated with AD-type dementia.

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