

## Effects of *Melissa officinalis* L. (Lemon Balm) Extract on Neurogenesis Associated with Serum Corticosterone and GABA in the Mouse Dentate Gyrus

Dae Young Yoo · Jung Hoon Choi ·  
Woosuk Kim · Ki-Yeon Yoo · Choong Hyun Lee ·  
Yeo Sung Yoon · Moo-Ho Won · In Koo Hwang

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**Abstract** Lemon balm, leaves of *Melissa officinalis* L., has been used for anti-anxiety and spasmolytics. We observed the extract of *Melissa officinalis* L. (MOE) on cell proliferation and neuroblast differentiation in the hippocampal dentate gyrus (DG) of middle-aged mice (12 months of age) using Ki67 and doublecortin (DCX), respectively. We also observed changes in corticosterone, GAD67 and GABA-transaminase (GABA-T) to check their possible mechanisms related to neurogenesis. We administered 50 or 200 mg/kg MOE to the animals once a day for 3 weeks. For labeling of newly generated cells, we also

administered 5-bromodeoxyuridine (BrdU) twice a day for 3 days from the day of the first MOE treatment. Administration of 50 or 200 mg/kg MOE dose-dependently increased Ki67 positive nuclei to 244.1 and 763.9% of the vehicle-treated group, respectively. In addition, 50 or 200 mg/kg MOE significantly increased DCX positive neuroblasts with well-developed (tertiary) dendrites. Furthermore, MOE administration significantly increased BrdU/calbindin D-28 k double labeled cells (integrated neurons into granule cells in the DG) to 245.2% of the vehicle-treated group. On the other hand, administration of MOE reduced corticosterone levels in serum and decreased GABA-T levels in the DG homogenates. These results suggest that MOE increases cell proliferation, neuroblast differentiation and integration into granule cells by decreasing serum corticosterone levels as well as by increasing GABA levels in the mouse DG.

D. Y. Yoo · W. Kim · Y. S. Yoon · I. K. Hwang (✉)  
Department of Anatomy and Cell Biology, College of Veterinary Medicine, and Research Institute for Veterinary Science, Seoul National University, Seoul 151-742, South Korea  
e-mail: vetmed2@snu.ac.kr

J. H. Choi  
Department of Anatomy, College of Veterinary Medicine, Kangwon National University, Chuncheon 200-701, South Korea

K.-Y. Yoo  
Institute of Natural Medicine, Hallym University, Chuncheon 200-702, South Korea

C. H. Lee · M.-H. Won (✉)  
Department of Neurobiology, School of Medicine, Kangwon National University, Chuncheon 200-701, South Korea  
e-mail: mhwon@kangwon.ac.kr

C. H. Lee  
Laboratory of Veterinary Pharmacology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, South Korea

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### Introduction

It has been reported that the brain has ability to heal itself after some injury or as a result of a disease [1, 2]. Neurogenesis occurs actively in the injured brain, however, only several regions have neurogenic ability [3]. Among these regions, the hippocampal dentate gyrus (DG) is one of most important regions because the hippocampus is mainly related to recent memory and several neurological disorders including Alzheimer's disease and stroke [4–7]. Cells are generated in a narrow band of the subgranular zone of the DG, and they migrate into the granule cell layer

of the DG within which new neurons are produced [8]. Some researchers showed strategies to promote differentiation of neuronal progenitors into mature functional neurons in the brain induced by Alzheimer's disease and stroke [2, 4].

There are many factors to affect neurogenesis in the brain. Reactive oxygen species, one of these factors, can potently inhibit neurogenesis, particularly neural progenitor cell proliferation and decrease neuronal proliferation [9]. We need to search new materials to enhance neurogenesis. *Melissa officinalis* L. called lemon balm has been used in traditional medicine to induce nerve calming and spasmolytic effects [10–13]. The extract of *Melissa officinalis* L. (MOE) contains phenolic compounds such as flavonoids and phenolic acids that may scavenge free radicals [14, 15], and it contains rosmarinic acid, the triterpenoids ursolic acid and oleanolic acid that inhibit GABA-transaminase (GABA-T, EC 2.6.1.19) [16], which is enzyme for GABA degradation. In addition, MOE has protective effects on hypergen peroxide induced toxicity in PC12 cells [17].

MOE is able to inhibit GABA-T activity, and treatment with GABA agonists increases maturation and survival of proliferating cells [18, 19]. We examined, therefore, the possibility of MOE as a neurogenic substance using Ki67, which is an endogenous marker for cell proliferation and detects all active cell cycles except for early G1 at sacrifice period [20], and using DCX, which is a maker for neuroblasts and expressed in immature neurons and migratory neuroblasts [21, 22]. We also examined changes in serum corticosterone levels, GABA-T and GAD67 (EC 4.1.1.15) protein levels in the mouse DG to elucidate possible mechanism of MOE on neurogenesis.

## Materials and Methods

### Preparation of MOE

For MOE, leaves of *Melissa officinalis* L. (1,000 g) were obtained from a local grocery store Poong Mul Si Jang, Chuncheon, South Korea. They were chopped and blended using a Waring blender and boiled with 2 L of 20% ethanol at 80°C for 2 h. The insoluble materials were removed through centrifugation at 10,000 × g for 30 min, and the resulting supernatant was concentrated and freeze-dried to yield a white powder (Yield: 11.0%).

### Experimental Animals

The progeny of male C57BL/6 J mice were purchased from the Jackson Laboratory Co. Ltd (Bar Harbor, ME).

Twelve-month-old mice were used in this study. The animals were housed in a conventional state under adequate controlled temperature (22°C), humidity (55%), 12 h light/12 h dark cycle, and provided with free access to food and tap water. The procedures for the care and handling of animals conformed to guidelines that are in compliance with current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996). All of the experiments were conducted in a way as to minimize the number of animals used and the suffering caused by the procedures used in the present study.

### Treatment with MOE and BrdU

Mice were equally divided into 3 groups: vehicle (distilled water)-treated group (vehicle-group), 50 and 200 mg/kg MOE-treated groups (MOE-group) ( $n = 12$  in each group) [23]. Vehicle or MOE were orally administered to mice using a feeding needle once a day for 21 days and the animals were sacrificed at 2 h after the last MOE administration. We adopted these schedules because DCX is exclusively expressed in immature neurons from only 1 to 28 days of cell age [24, 25]. In order to determine the integration of new neurons generated in the adult brain, the animals in vehicle-group ( $n = 5$ ) and 200 mg/kg MOE-group ( $n = 5$  in each group) were treated with 50 mg/kg 5-bromodeoxyuridine (BrdU, Sigma, St. Louis, MO) twice a day for 3 days from the day of the first MOE treatment.

### Blood Sampling and Tissue Processing for Histology

For blood sampling, histology and western blot, the animals ( $n = 12$  in each group) were anesthetized with intraperitoneal injection of 30 mg/kg chloral hydrate (Sigma), and blood samples were collected from each mouse by cardiac puncture. Serum was separated from the blood by centrifugation at 1,100 g for 15 min at 4°C and was kept at -80°C until analysis. Thereafter, the animals were perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and postfixed in the same fixative for 4 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thirty- $\mu\text{m}$ -thick brain sections in coronal plane were serially cut using a cryostat (Leica, Wetzlar, Germany). The sections were collected into six-well plates containing PBS for further process.

### Measurements of Serum Corticosterone Levels

Serum (50  $\mu\text{L}$ ) was added to 5 mL of methylene chloride and incubated at room temperature for 10 min. After

filtration with cheesecloth, the mixture was combined with 2.5 mL of fluorescence reagent (7:3, sulfuric acid/absolute ethanol), vortexed vigorously and incubated for 30 min at room temperature. After centrifugation, the lower layer was measured using a spectrophotometer (excited wavelength, 475 nm; emission wavelength, 530 nm).

### Immunohistochemistry

To obtain the accurate data for immunohistochemistry, the free-floating sections were carefully processed under the same conditions. The tissue sections were selected between -1.46 mm and -2.46 mm posterior to the bregma in reference to the mouse atlas [26] for each animal. The sections were sequentially treated with 0.3% hydrogen peroxide ( $H_2O_2$ ) in PBS for 30 min and 10% normal goat or rabbit serum in 0.05 M PBS for 30 min. They were next incubated with diluted rabbit anti-Ki67 (1:1,000, Abcam, Cambridge, UK) or goat anti-DCX antibody (1:50, Santa-Cruz Biotechnology, Santa Cruz, CA) overnight at room temperature and subsequently exposed to biotinylated goat anti-rat IgG, rabbit anti-goat or goat anti-rabbit IgG (diluted 1:200, Vector, Burlingame, CA) and streptavidin peroxidase complex (diluted 1:200, Vector). Then, the sections were visualized with reaction to 3,3'-diaminobenzidine tetrachloride (Sigma, St. Louis, MO) in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides.

### Double Immunofluorescence for BrdU/CB

The sections were processed by double immunofluorescence staining using rabbit anti-CB (calbindin D-28 k, 1:1,000, Chemicon international, Temecula, CA)/rat anti-BrdU (1:200, BioSource International, Camarillo, CA). For BrdU immunostaining, DNA was first denatured by incubating the sections in 50% formamide/2 × standard saline citrate at 65°C for 2 h and in 2 N HCl at 37°C for 30 min. They were incubated in the mixture of antisera overnight at 4°C. After washing with PBS, they were then incubated in a mixture of both FITC-conjugated anti-rat IgG (1:200; Jackson ImmunoResearch, West Grove, PA) and Cy3-conjugated anti-rabbit IgG (1:600; Jackson ImmunoResearch). The immunoreactions were observed under a BX51 light microscope (Olympus, Tokyo, Japan) attached with fluorescence lamp power supply (Olympus).

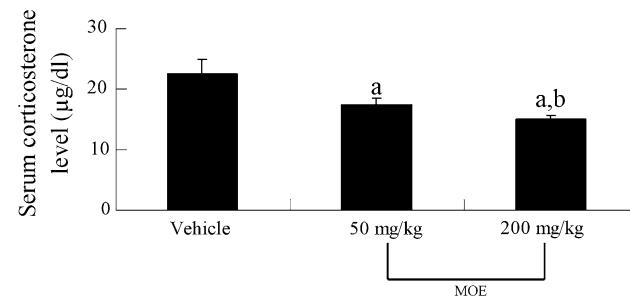
### Western Blot Analysis

To confirm changes in GAD67 and GABA-T levels in the hippocampus, 5 animals in each group were sacrificed and used for western blot analysis. After sacrificing them and removing the brain, DGs were dissected out using PixCell

II system (Arcturus Engineering). The tissues were homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol bis (2-aminoethyl Ether)-N,N,N',N' tetraacetic acid (EGTA) (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylenediamine tetraacetic acid (EDTA) (pH 8.0), 15 mM sodium pyrophosphate, 100 mM  $\beta$ -glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). After centrifugation, the protein level was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL). Aliquots containing 20  $\mu$ g of total protein were boiled for 5 min in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto polyacrylamide gels. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with mouse anti-GABA-T [27–29] or mouse anti-GAD67 (1:50) peroxidase-conjugated anti-goat IgG or anti-mouse IgG (Sigma) and an enhanced luminol-based chemiluminescent (ECL) kit (Pierce Chemical). The result of the western blot analysis was scanned, and densitometric analysis for the quantification of the bands was done using Scion Image software (Scion Corp., Frederick, MD), which was used to count relative optical density (ROD). These data were normalized against  $\beta$ -actin.

### Quantification of Data

The measurement of Ki67, DCX and BrdU/CB positive cells in all groups was performed using an image analyzing system equipped with a computer-based CCD camera (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ). In addition, images of all DCX-immunoreactive structures were taken from the DG through a BX51 light microscope

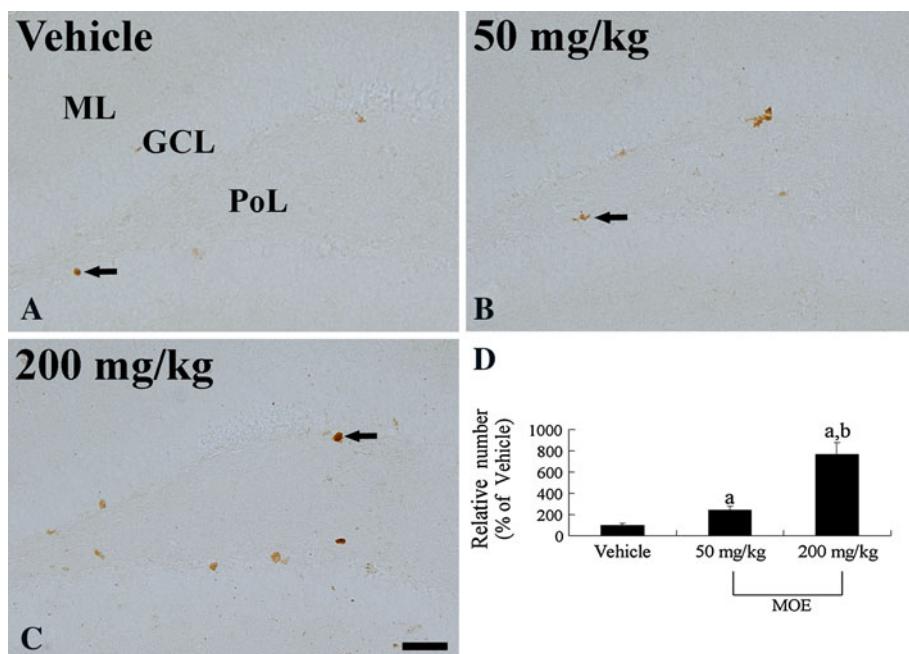


**Fig. 1** Serum corticosterone levels in the vehicle-, 50 and 200 mg/kg MOE-groups ( $n = 7$  per group; <sup>a</sup> $P < 0.05$ , versus vehicle-treated group; <sup>b</sup> $P < 0.05$ , versus 50 mg/kg MOE-treated group). The bars indicate the means  $\pm$  SEM

**Fig. 2** Immunohistochemistry for Ki67 in the DG of the vehicle- (a), 50 (b) and 200 mg/kg (c) MOE-groups. In all the groups, Ki67 positive nuclei (arrows) are detected in the DG. Note that Ki67 positive nuclei are abundant in the MOE-group compared to those in the vehicle-group. GCL, granule cell layer; ML molecular layer, PoL polymorphic layer.

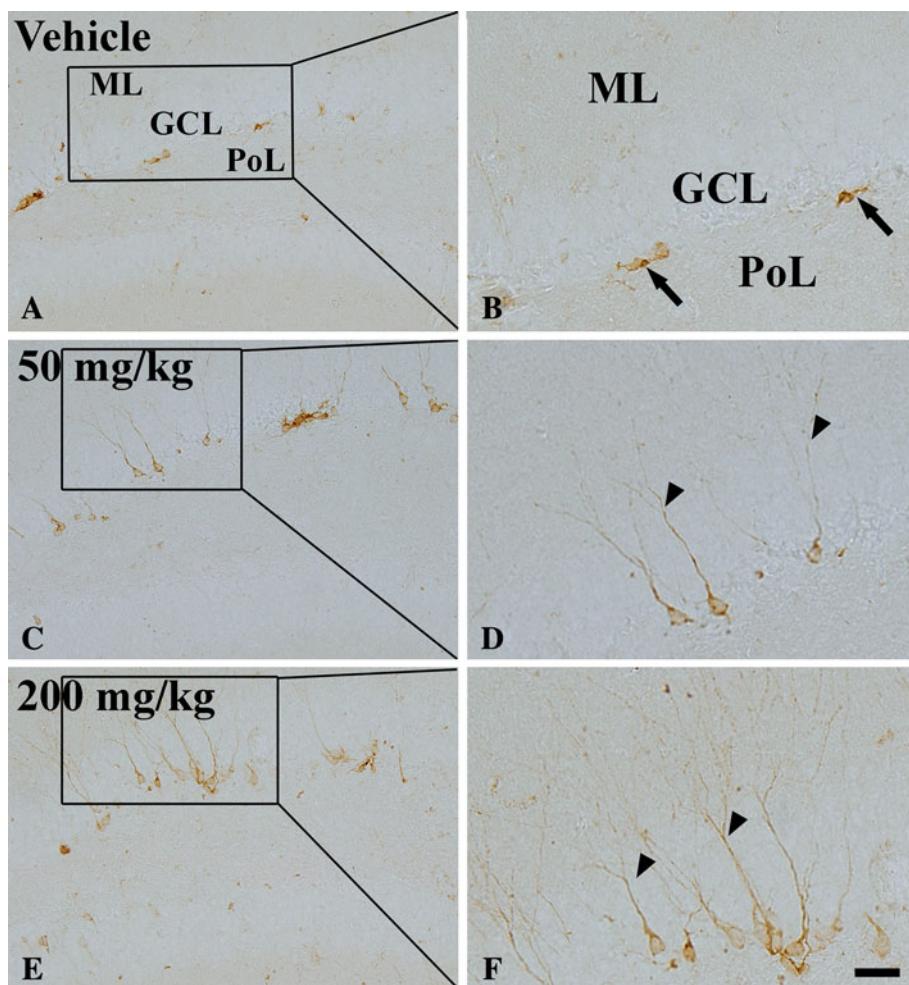
Bar = 50 µm (a, b and c).

d Relative number of Ki67 positive nuclei in the DG of the vehicle-, 50 and 200 mg/kg MOE-groups ( $n = 7$  per group;  $^aP < 0.05$ , versus the vehicle-group;  $^bP < 0.05$ , versus the 50 mg/kg MOE-group). The bars indicate the means  $\pm$  SEM



**Fig. 3** Immunohistochemistry for DCX in the DG of the vehicle- (a and b), 50 (c and d) and 200 mg/kg (e and f) MOE-groups. DCX positive neuroblasts (arrows) are found in the vehicle-group. In the 50 and 200 mg/kg MOE-groups, DCX positive neuroblasts have well-developed processes, which extend to the molecular layer (ML) of the DG. GCL, granule cell layer, PoL polymorphic layer.

Bar = 50 µm (a, c and e), 25 µm (b, d and f)



(Olympus, Tokyo, Japan) equipped with a digital camera (DP71, Olympus) connected to a PC monitor. Dendritic complexity of DCX positive cells was traced using camera lucida at  $100 \times$  magnification (Neurolucida; Micro-BrightField, Williston, VT). DCX positive cells were separated into 2 categories according to dendritic complexity; (1) lack dendrites but have immature dendrites (primary or secondary branches which do not extend into the outer molecular layer) and (2) have mature dendrites (with tertiary branches which extend into the outer molecular layer). Then, the DCX positive cells in the DG in each section were counted using Optimas 6.5 software (Cyber-Metrics). Average count from the sections of all mice was presented. A ratio of the count was calibrated as % of vehicle group.

#### Statistical Analysis

The data shown here represent the means of experiments performed for each experimental area. Differences among the means were statistically analyzed by one-way analysis of variance followed by Tukey's multiple range method in order to elucidate differences between vehicle- and MOE-groups.

## Results

### Effects of MOE on Serum Corticosterone Levels

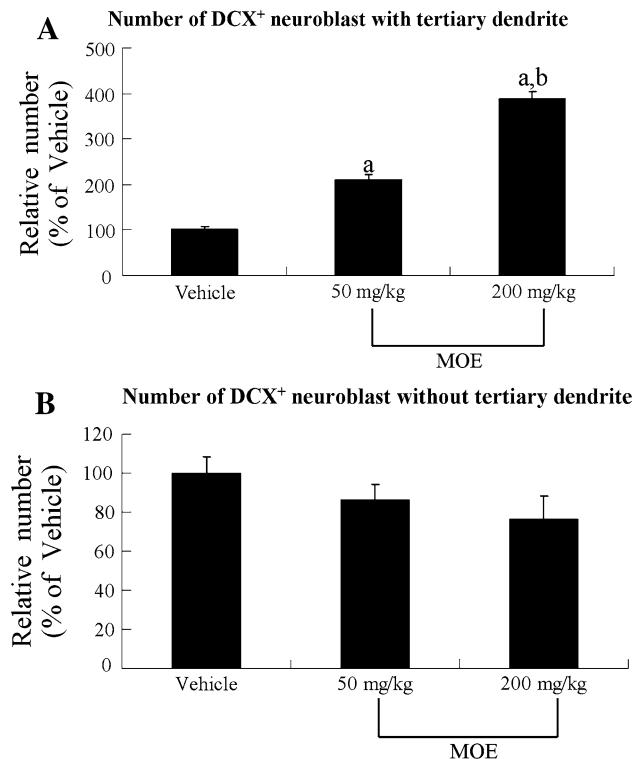
In the vehicle-group, serum corticosterone level was 22.4  $\mu\text{g}/\text{dL}$  (Fig. 1). However, in the 50 and 200 mg/kg MOE-group, serum corticosterone level was significantly decreased dose-dependently and was 17.4 and 14.9  $\mu\text{g}/\text{dL}$ , respectively (Fig. 1).

### Effects of MOE on Cell Proliferation

We observed the cell proliferation in the subgranular zone of the DG using Ki67 immunohistochemistry. In the vehicle-group, Ki67 positive nuclei were detected in a few cells in the subgranular zone of the DG (Fig. 2a), whereas, in the MOE-groups, Ki67 positive nuclei were dose-dependently increased in the subgranular zone of the DG (Fig. 2b and c): The number of Ki67 positive nuclei in the 50 and 200 mg/kg MOE-group was 244.1% and 763.9% compared to that in the vehicle-group, respectively (Fig. 2D).

### Effects of MOE on Neuroblast Differentiation

We observed the neuroblast differentiation in the subgranular zone of the DG using DCX immunohistochemistry. In



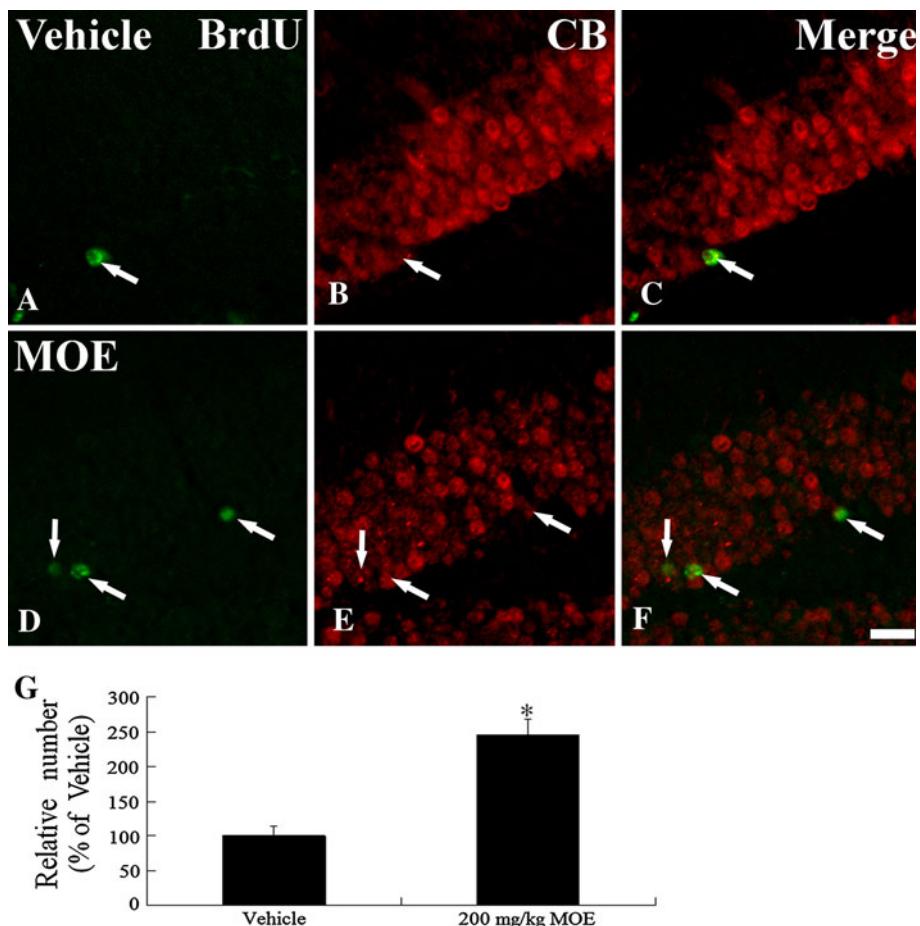
**Fig. 4** Relative number of DCX-immunoreactive neuroblasts with/without tertiary dendrites in the DG ( $n = 7$  per group; <sup>a</sup> $P < 0.05$ , versus the vehicle-group; <sup>b</sup> $P < 0.05$ , versus the 50 mg/kg MOE-group). The bars indicate the means  $\pm$  SEM

the vehicle-group, DCX-immunoreactive neuroblasts were detected in the subgranular zone of the DG with poorly-developed dendrites (Fig. 3a and b). In the 50 and 200 mg/kg MOE-group, DCX-immunoreactive neuroblasts were also detected in the subgranular zone of dentate gyrus: In these groups, DCX-immunoreactive neuroblasts had poorly-developed dendrites or well-developed tertiary dendrites (Fig. 3c, d, e and f). Especially, the number of DCX-immunoreactive neuroblasts with tertiary dendrites in the 50 and 200 mg/kg MOE-group was significantly increased dose-dependently to 211.4 and 387.4% of the vehicle-group, respectively (Fig. 4). However, the number of DCX-immunoreactive neuroblast without tertiary dendrites in each group was slightly decreased to 86.3 and 76.4% of the vehicle-group, respectively (Fig. 4).

### Effects of MOE on Integration of Newly Generated Cells into Granule Cells

In the vehicle-group, few BrdU positive cells were detected in the DG (Fig. 5a) and few BrdU/CB colabeled cells were detected in the dentate gyrus (Fig. 5a–c and g). In the 200 mg/kg MOE-treated group, BrdU/CB colabeled cells

**Fig. 5** Double immunofluorescence micrographs for BrdU (green) and CB (red) in the DG of the vehicle- (**a–c**) and 200 mg/kg MOE (**d–f**) groups. Numbers of BrdU/CB positive cells (arrows) in the MOE-group are more than those in the vehicle-group. Bar = 25  $\mu$ m. **g** Relative number of BrdU/CB-colabeled cells in the DG of the vehicle- and 200 mg/kg MOE-groups ( $n = 5$  per group; \* $P < 0.05$ , significantly different from the vehicle-group). The bars indicate the means  $\pm$  SEM. For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article



were significantly increased and observed 245.2% of vehicle-treated group (Fig. 5d–f and g).

#### Effects of MOE on GAD67 and GABA-T levels

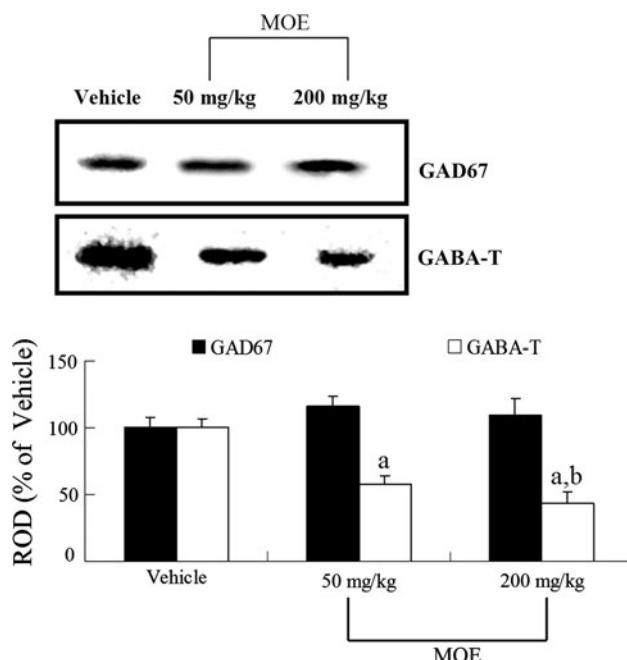
GAD67 protein levels were similar in all the MOE-groups compared to that in the vehicle-group. However, GABA-T protein level in each MOE-group was significantly decreased to 57.3 and 43.5% of the vehicle-group, respectively (Fig. 6).

#### Discussion

It was reported that high dose of MOE (600 mg/kg) increased calmness and reduced alertness in human study [10]. In addition, MOE has anti-anxiety effects by reducing brain concentrations of corticosterone during open field and elevated plus maze tests in mice [23]. In our present study, we also observed that MOE treatment significantly reduced the serum corticosterone levels in mice compared

to that in the vehicle-group. Corticosteroid hormones regulate proliferation of newborn cells and survival/cell death of mature granule cells in the DG of the adult brain [30–32]. In addition, corticosterone regulates brain-derived neurotrophic factor, one of major factors on stimulating neurogenesis, by stimulating action in the DG [33].

In this study, we observed the effects of MOE on the cell proliferation and neuroblast differentiation in the subgranular zone of the DG. The administration of 50 and 200 mg/kg MOE dose-dependently increased Ki67 positive nuclei to 244.1 and 763.9% of the vehicle-group, respectively. In addition, 50 and 200 mg/kg MOE significantly increased DCX positive neuroblasts with well-developed (tertiary) dendrites compared to the vehicle-group. There are several reports that some extracts promote the neurogenesis in the DG. *Ginkgo biloba* extract increases cell proliferation in the DG of a mouse model of Alzheimer's disease [34] as well as healthy mice [35]. In addition, we reported that aqueous extract from *Platycodon grandiflorum* significantly increased cell proliferation and neuroblast differentiation in the subgranular zone of the DG in the middle-aged mice [36].



**Fig. 6** Western blot analyses of GAD67 and GABA-T in the DG of the vehicle-, 50 and 200 mg/kg MOE-groups. The relative optical density (ROD) of the immunoblot bands are indicated as percent values versus the vehicle-group ( $n = 5$  per group; <sup>a</sup> $P < 0.05$ , versus the vehicle-group; <sup>b</sup> $P < 0.05$ , versus the 50 mg/kg MOE-group). The bars indicate the means  $\pm$  SEM

In the present study, the animals were administered BrdU for the first 3 days after MOE treatment and sacrificed at 28 days after BrdU treatment. We observed that the administration of MOE significantly increased the integration into mature granule cells, showing the increase of 245% in the DG. This protocol was adapted because newly generated cells in the normal brain project their axons into CA3 and hilar area at 10–11 days after birth, and, finally, spine formations are peaked during the first 3–4 weeks after birth [37]. Furthermore, we examined that MOE administration significantly increased BrdU/CB double labeled cells (integrated neurons into granule cells in the DG) to 245% of the vehicle-group.

In this study, we observed the levels of GAD67 and GABA-T, which regulates the synthesis and breakdown of GABA, respectively [38] after MOE administration to observe the correlation between cell proliferation, neuroblast differentiation and GABA levels in the mouse DG. We observed significant decrease of GABA-T level in the DG after MOE administration, whereas any significant changes in GAD67 protein level were not observed in the mouse DG after MOE administration. This suggests that GABA levels are increased in the DG because of reduction in GABA breakdown. It was reported that aqueous MOE showed high inhibitory affinity against GAD and GABA-T in an in vitro study [16, 39]. Inhibition of GABA-T

increased the available amount of GABA in the brain [16] and the increased GABA levels might potentiate the survival of newly born cells because proliferating cells in the DG shortly after birth express GABA<sub>A</sub> receptors and respond to GABA [19, 40–42]. This is supported by previous studies that treatment with GABA agonists increases maturation and survival of proliferating cells [18, 19]. In addition, administration of eszopiclone, a GABA agonist, enhances cell proliferation and survival of newborn cells [43].

In conclusion, administration of MOE significantly increases cell proliferation and neuroblast differentiation in the mouse DG with reduction of corticosterone levels in serum and increase of GABA levels in the mouse DG.

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