FERULIC ACID INDUCES NEURAL PROGENITOR CELL PROLIFERATION *IN VITRO* AND *IN VIVO*

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Abstract—Ferulic acid (4-hydroxy-3-methoxycinnamic acid; FA) is a plant constituent and is contained in several medicinal plants for clinical use. In this paper, we investigated the effects of FA on the proliferation of neural stem/progenitor cells (NSC/NPCs) *in vitro* and *in vivo*. FA significantly increased the proliferation of NSC/NPCs cultured from the telencephalon of embryonic day-14 rats, and increased the number and size of secondary formed neurospheres. An *in vitro* differentiation assay showed that FA did not affect the percentage of either neuron-specific class III β-tubulin (Tuj-1)-positive cells or glial fibrillary acidic protein (GFAP)-positive cells in the total cell population. Oral administration of FA increased the number of newly generated cells in the dentate gyrus (DG) of the hippocampus of corticosterone (CORT)-treated mice, indicating that FA enhances the proliferation of adult NSC/NPCs *in vivo*. We also found that oral administration of FA increased cAMP response element binding protein (CREB) phosphorylation and brain-derived neurotrophic factor (BDNF) mRNA level in the hippocampus of CORT-treated mice, and ameliorated the stress-induced depression-like behavior of mice. These novel pharmacological effects of FA may be useful for the treatment of mood disorders such as depression. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: corticosterone, stress, depression, forced swimming test, CREB, BDNF.

Neural stem cells (NSCs) and neural progenitor cells (NPCs) are multipotential progenitor cells that can give rise to both neurons and glia in the fetal and adult CNS (Reynolds and Weiss, 1996; Gage, 2000). The dentate gyrus (DG) of the hippocampus is one of the specific areas where there is ongoing neurogenesis in the adult mammalian brain (Altman and Das, 1965; Eriksson et al., 1998). NSC/NPCs in the subgranular zone (SGZ) of the hippocampus give rise to mature neurons that migrate into the granule cell layer (Hastings and Gould, 1999). Adult hippocampal neurogenesis is influenced by environmental and pharmacological stimuli (Gage, 2000; Duman et al., 2001; Kempermann, 2002). For example, hippocampal neurogenesis is increased by exercise, an enriched environment, and hippocampal-dependent learning (Gould et al., 1999; Nilsson et al., 1999; van Praag et al., 1999). On the other hand, it has been reported that stress and glucocorticoids reduce NSC/NPC proliferation, giving rise to anatomical abnormalities and pathological responses to stress (Cameron and Gould, 1994; Gould et al., 1997). Furthermore, stress-induced reductions in NSC/NPC proliferation and neurogenesis in the hippocampus can also be reversed with antidepressant treatments, and hippocampal neurogenesis seems to be required for the behavioral recovery effects of antidepressant treatment (Czeh et al., 2001; Santarelli et al., 2003). These observations suggest that the degradation of hippocampal neurogenesis is closely associated with the pathology of mood disorders such as major depression. Therefore, regulation of neurogenesis by NSC/NPCs is anticipated as a therapeutic target for mood disorders.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid; FA) (Fig. 1A) is a plant constituent that is known to exhibit strong effects on scavenging free radicals and antioxidant capacity (Srinivasan et al., 2007). Although FA is reported to have many pharmacological effects such as anti-inflammatory, anticancer, anti-diabetic, anti-atherogenic and neuroprotective (Mukhopadhyay et al., 1982; Kawabata et al., 2000; Balasubashini et al., 2004; Sultana et al., 2005; Yogeeta et al., 2006), its biological activities on the CNS remain largely unknown. In the present study, we examined whether FA could regulate the properties of NSC/NPCs *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Cell culture

Primary neurospheres were isolated from the telencephalon of Wistar rats at E14.5 and cultured in neurosphere proliferation media consisting of Dulbecco's Modified Eagle's Medium (DMEM): F12 (Sigma, St. Louis, MO, USA) supplemented with 1% (v/v) N2 Supplement (Invitrogen, Carlsbad, CA, USA), 20 ng/ml epidermal growth factor (EGF; PEPROTECH, Rocky Hill, NJ, USA), and 20 ng/ml fibroblast growth factor 2 (FGF2; PEPROTECH). After 7 days in culture, neurospheres that formed were gathered and dissociated into a single cell suspension by pipetting. Dissociated

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Abbreviations: BDNF, brain-derived neurotrophic factor; CMS, chronic mild stresses; CORT, corticosterone; CREB, cAMP response element binding protein; DG, dentate gyrus; EGF, epidermal growth factor; FA, Ferulic acid; FGF2, fibroblast growth factor 2; FS, forced swimming; GCL, granular cell layer; GFAP, glial fibrillary acidic protein; NPC, neural progenitor cell; NSC, neural stem cell; pCREB, phosphorylated CREB; SGZ, subgranular zone; SNRI, serotonin and norepinephrine reuptake inhibitor; Tuj-1, neuron-specific class III β-tubulin.
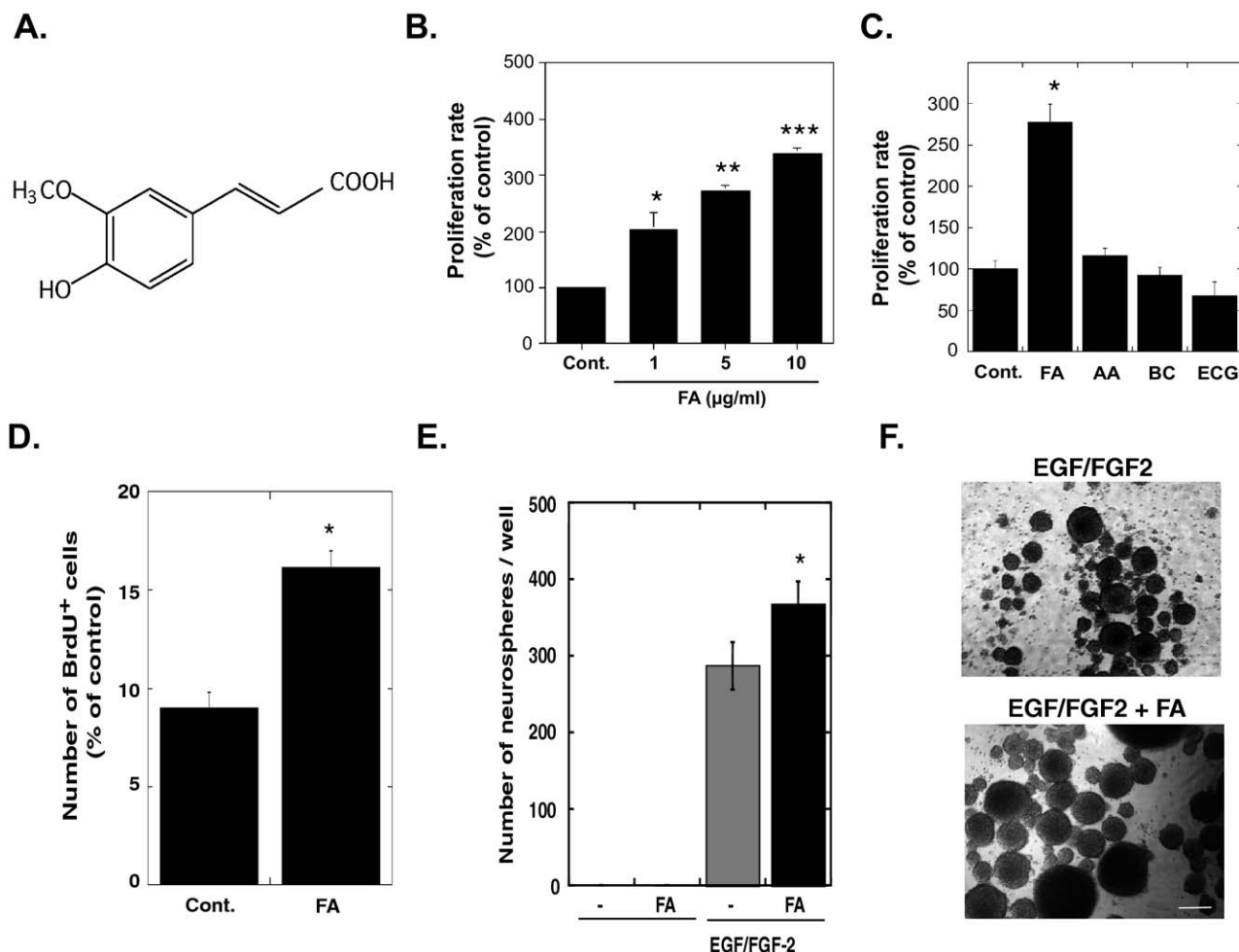
Fig. 1. FA stimulates NSC/NPC proliferation. (A) Chemical structure of ferulic acid (FA; 4-hydroxy-3-methoxycinnamic acid). (B) Effect of FA on the proliferation of cultured NSC/NPCs. Dissociated NSC/NPCs were cultured on a poly-L-lysine-coated 96-well plate with the indicated FA concentration for 2 d. Cell proliferation was measured using an Alamar Blue assay. Each column represents the mean±SEM ($n=3$). * $P<0.05$, ** $P<0.01$, *** $P<0.0005$ versus control. Similar results were obtained in three independent experiments. (C) Effect of anti-oxidants on cell proliferation of NSC/NPCs. Dissociated NSC/NPCs were cultured on a poly-L-lysine-coated 96-well plate with ascorbic acid (AA; 10 μg/ml), baicalein (BC; 10 μg/ml), epigallocatechin gallate (ECG; 10 μg/ml) or FA (10 μg/ml). Cell proliferation was measured using an Alamar Blue assay. Each column represents the mean±SEM ($n=3$). * $P<0.0001$ versus control. Similar results were obtained in three independent experiments. (D) Effect of FA on BrdU-incorporation in cultured NSC/NPCs. Dissociated NSC/NPCs were cultured on poly-L-lysine-coated 8-well chamber slides with or without 10 μg/ml FA for 5 h and were subsequently incubated with BrdU (5 μM) for 2 h. After treatment, cells were fixed, immunostained with anti BrdU antibody, and counterstained with propidium iodide. BrdU-positive cells were counted in 14 randomly selected fields from three different chambers. The percentage of BrdU-positive cells was calculated as the number of BrdU-positive cells divided by the total number of cells (propidium iodide-stained cells). Each column represents the mean±SEM ($n=14$). * $P<0.0001$ versus control. Similar results were obtained in two independent experiments. (E) Effect of FA on secondary neurosphere formation. Dissociated NSC/NPCs (0.5×10^5 cells/well on a 24-well non-coated plate) were incubated for 11 d with or without FA (10 μg/ml) or growth factors (EGF/FGF2). Values represent the mean±SEM ($n=6$) * $P<0.05$ versus EGF/FGF2-treated cells. Similar results were obtained in three independent experiments. (F) Representative microphotographs of formed neurospheres in the absence (upper picture) or presence (lower picture) of FA (10 μg/ml). FA increased the size of formed neurospheres.

cells were used as cultured NSC/NPCs for proliferation, differentiation and secondary neurosphere formation assays.

In vitro cell proliferation assay (Alamar blue assay)

For *in vitro* cell proliferation assay, we utilized Alamar blue assay. Alamar blue (Resazurin), a non-fluorescent indicator dye, is converted to bright red–fluorescent resorufin via the reduction reactions of metabolically active cells. The amount of fluorescence produced is proportional to the number of living cells. Cultured NSC/NPCs (1×10^5 cells) were plated onto a poly-L-lysine-coated 96-well plate without growth factors. After 3 days in culture with or without FA (Sigma), Alamar Blue reagent (Alamar Bio-Sciences, Sacramento, CA, USA) was added to each well. The plate was incubated further for 2 h and then the fluorescence intensity in each well was recorded with a microplate fluorescence reader (Fluoroskan II; Labsystems, Helsinki, Finland) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The cell proliferation rate was expressed as the relative fluorescence intensity. Effect of antioxidants (ascorbic acid, baicalein and epigallocatechin gallate) or cinnamic acid derivatives (3-methoxycinnamic acid, 3-hydroxycinnamic acid, 3,4-dimethoxycinnamic acid, 3-hydroxy-4-methoxycinnamic acid, 2,3-dimethoxycinnamic acid, 2,4-dimethoxycinnamic acid, 2,5-dimethoxycinnamic acid,

and 4-hydroxycinnamic acid) on NSC/NPC proliferation was also tested by same protocol. Antioxidants and cinnamic acid derivatives were obtained from Sigma. FA and cinnamic acid derivatives were dissolved in EtOH. All were diluted to their final concentration in cultured media (final EtOH concentration is 0.5%). Vehicle consisted of 0.5% EtOH in culture media.

In vitro BrdU-incorporation assay

For *in vitro* BrdU-incorporation assay, cultured NSC/NPCs (5×10^5 cells) were plated onto poly-L-lysine coated eight-well chamber slides in DMEM supplemented with 1% (v/v) N2 Supplement. Cultured NSC/NPCs were incubated with or without FA for 5 h and subsequently maintained with 5 mM BrdU for 2 h. The cells were washed with phosphate-buffered saline (PBS) and incubated in 2N HCl at 37 °C for 35 min. After washing in PBS, they were incubated with anti-BrdU antibody (1:400) at 4 °C for 24 h in PBS containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100. After washing in PBS, they were then incubated at room temperature for 2 h in PBS containing 1% BSA plus Alexa Fluor 488 conjugated anti-mouse IgG secondary antibody (1:2000; Invitrogen). After immunostaining, cells were stained with propidium iodide (5 μg/ml) for 5 min. BrdU-positive cells were evaluated using a fluorescent microscope (Keyence, Osaka, Japan). BrdU-positive cells were counted in 12 randomly selected fields from three different chambers. Approximately 26,000 cells were scored for each treatment. The percentage of BrdU-positive cells was calculated as the number of BrdU-positive cells divided by the total number of cells (propidium iodide-stained cells).

Secondary neurosphere formation assay

For the secondary neurosphere formation assay, primary neurospheres were dissociated into a single cell suspension and cultured in neurosphere proliferation media containing EGF and FGF with or without FA for 11 days (0.5×10^5 cells/well on a 24-well non-coated plate). Total number of secondary neurospheres that formed in a 24-well plate was counted under an Olympus CK-40 microscope (Olympus, Tokyo, Japan). The number of neurospheres in each well was counted two to three times, and the average of counted score was used for evaluation.

In vitro differentiation assay

For the *in vitro* differentiation assay, NSC/NPCs (0.5×10^4 cells/well) were cultured on laminin/poly-L-ornithine-coated eight-well chambers with or without FA in growth factor-free medium for 7 days. For fluorescence immunostaining, cells were fixed with 4% paraformaldehyde for 30 min. To block nonspecific antibody binding, cells were incubated in a blocking solution containing 1% BSA and 0.3% Triton X-100 for 2 h. Cells were then incubated with the following primary antibody: mouse monoclonal anti-Tuj-1 (1:500: Covance the Development Services Company, Berkeley, CA, USA) and rabbit polyclonal anti-GFAP (1:500: Dako Cytomation, California Inc, Carpinteria, CA, USA) at 4 °C overnight followed by Alexa Fluor 488 conjugated anti-mouse IgG secondary antibody (1:1500; Invitrogen) and Alexa Fluor 594 anti-rabbit IgG secondary antibody (1:1500; Invitrogen). 4',6-Diamino-2-phenylindole (DAPI) was used as a fluorescent nuclear counterstain. Stained cultures were examined and photographed by fluorescence microscopy (Keyence, Osaka, Japan).

Animals

Adult (7 week-old) male ddY mice (Japan SLC, Hamamatsu, Japan) weighing 35–40 g were used for the *in vivo* experiments. Mice were housed individually under conditions of constant temperature (23 ± 2 °C) and humidity ($55 \pm 10\%$) with food and water available *ad libitum*, unless otherwise specified, and a 12 h light/ dark cycle (light at 8:00 h and dark at 20:00 h). All animal experiments were performed according to the Guidelines for Care and Use of Laboratory Animals at the Kitasato Institute and Kitasato University, and conformed to the US National Institutes of Health guidelines on the ethical use of animals. Every effort was made to minimize the number of animals used and their suffering.

Administration of CORT, FA, and BrdU

A daily subcutaneous injection of corticosterone (CORT) (40 mg/ kg; Sigma) in sesame oil or oil alone was given for 8 days. FA (50, 100 or 250 mg/kg) was suspended in distilled water, and administered orally using intragastric gavage during the same period of CORT administration. BrdU (100 mg/kg; Sigma) was injected intraperitoneally at day 6 and 8. Twenty-four hours after the last BrdU injection, mice were anesthetized with ether and perfused transcardially with cold PBS, followed by a cold 4% paraformaldehyde solution. Brains were collected and postfixed overnight in a 4% paraformaldehyde solution at 4 °C. Serial coronal sections (50 μm thickness) were obtained throughout the hippocampus (bregma -1.2 to -2.5 mm) using a vibratome (Technical Products International Inc., St. Louis, MO, USA), and sections were stored in PBS/NaN$_3$ at 4 °C until needed for subsequent experiments.

Immunohistochemistry

For analysis involving phosphorylated CREB (pCREB) immuno-histochemistry, free-floating sections were incubated in an antigen-activated solution (10 mM sodium citrate buffer, pH 9.0) for 10 min at 95 °C, followed by incubation in 3% H$_2$O$_2$/80% methanol for 40 min at room temperature (RT) to inactivate the endogenous peroxidase. After washing in PBS, sections were blocked for 3 h with 1% BSA in PBS containing 0.3% Triton X-100 (PBS-T), and then incubated for 1 day at 4 °C with rabbit anti-phospho-CREB (Ser133) antibody (1:100; Cell Signaling Technology, Inc., Danvers, MA, USA). The sections were subsequently rinsed in PBS-T, incubated for 2 h at RT with biotinylated anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA), and incubated for 2 h at RT with avidin–biotin complex using an ABC kit (Vector Laboratories). pCREB-positive cells were visualized by incubating sections with a diaminobenzidine (DAB) solution (Sigma). Images were collected using a Biozero BZ8000 microscope (Keyence).

For analysis concerning BrdU immunohistochemistry, free-floating sections were incubated in 50% formamide/2$\times$ saline sodium citrate (SSC) for 2 h at 65 °C, followed by a rinse in 2$\times$SSC. Sections were then incubated in 2 N HCl for 30 min at 37 °C to denature double-stranded DNA, and rinsed in 0.1 M borate buffer (pH 8.5). After blocking for 2 h with 1% BSA in PBS-T, sections were incubated overnight at 4 °C with rat anti-BrdU monoclonal antibody (1:10; Chemicon, Temecula, CA, USA). After rinsing in PBS-T, sections were incubated for 2 h at RT with biotinylated horse anti-mouse IgG (1:200; Vector Laboratories), and incubated for 2 h at RT with the ABC kit. BrdU-positive cells were visualized by incubating sections with DAB solution (Sigma). BrdU-labeled cells throughout the rostrocaudal extent of the DG were counted in every sixth section, and the total number of BrdU-labeled cells was calculated by multiplying the count. Images were collected using a Biozero BZ8000 microscope (Keyence).

In situ hybridization (ISH)

ISH using biotin-labeled cRNA probes and tyramide signal amplification was performed using a modification of the method of van de Corput and Grosveld (2001). A 298-bp fragment encompassing nucleotides 975–1272 in the mice sequence (GenBank access number EF125669) for brain-derived neurotrophic factor (BDNF) was amplified using polymerase chain reaction (PCR) and mice brain cDNA. The sequences of the BDNF-specific primers are as

follows: sense, 5′-AGCCTCCTCTGCTCTTTCTGCTGGA-3′, and antisense, 5′-TTCCGACGTCCCCGTATCTGTTTTC-3′. The T7 RNA polymerase promoter was ligated to the PCR products using a Lig`nScribeTM No-Cloning Promoter Addition Kit (Ambion, Inc., Austin, TX, USA) according to the manufacturer's protocol. Biotin-labeled cRNA probes were prepared from T7-tailed PCR products by *in vitro* transcription using T7 RNA polymerase. Sections were hybridized overnight at 55 °C in a DAKO mRNA ISH solution (DAKO) containing 1 μg/ml of biotin-labeled cRNA probes. The hybridized signal was amplified using a TSA System (PerkinElmer, Waltham, MA, USA) and ABC kit (Vector Laboratories) according to the manufacturer's protocol. The hybridized signal was detected by incubating sections with a DAB solution (Sigma). No labeling was observed in any region of the brain in a negative control experiment with a BDNF cRNA sense probe, confirming the specific hybridization of the BDNF cRNA antisense probe (data not shown).

Stress-induced depression-like model mice

The stress-induced depression-like model mice (Ito et al., 2006) were prepared by a combination of modified forced swimming (FS) twice with 11 day interval (Detke et al., 1997; Porsolt, 1997) and chronic mild stress (CMS) (Willner et al., 1987; Monleon et al., 1995; Solberg et al., 1999). Briefly, the mice were individually placed into 5 L glass beakers (height 27 cm, diameter 18 cm) filled with 4 L of water (23±1 °C) for 15 min. The beakers were separated by non-transparent panels to prevent the mice from seeing each other. After 15 min in the water, the mice were removed and dried with hair dryer before being returned to their home cages. The mice were then separated into groups by measuring the duration of immobilities for the first 5 min of FS to minimize the variability of immobility among the groups. A mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. After 2 days, the mice were exposed to CMS, which consisted of three different stress situations: tilting of the cage twice 30 degrees from the horizontal (CMS 1), pouring 200 ml of water onto the sawdust bedding of the cage (CMS 2), and shaking the cages at 200 rpm using a Green S. Seriker II device (Vision Scientific, Kyunggi, Korea) (CMS 3). These stress situations were applied in 24 h intervals (see schematic Fig. 4A). The mice were then placed again into water at 60 min after the final treatment of the drug, and the total duration of immobility during a 5-min forced swimming test (FST) was measured. Forced swimming test was videotaped, and analyzed by an experienced pharmacologist, who was blind to the treatment.

Open field test (OFT)

The spontaneous locomotor activity of mice was measured by OFT. Mice were orally administered FA (0, 50, 100, 250 mg/kg) for 11 days. One day after the last administration, mice were placed individually in an opaque open field box (40×40×40 cm^3) and allowed to move freely for 5 min. Individual total distance and duration of movement were measured during the 5 min using a video tracking system (EthoVision; Noldus, Wageningen, Netherlands). This behavioral experiment was carried out between 13:00 h and 16:00 h.

Statistical analysis

Results were analyzed by a one-way analysis of variance (ANOVA) followed by Fisher's protected least square difference (PLSD) test or Student's *t*-test with STATview 5.0 software (SAS Institute, Cary, NC, USA). *P*-values less than 0.05 ($P<0.05$) are considered indicative of significance.

RESULTS

FA induces the proliferation of cultured NSC/NPCs

To identify novel compounds that can induce proliferation of NSC/NPCs, we screened 74 kinds of medicinal herb-derived compounds using an *in vitro* proliferation assay (unpublished data). Dissociated neurosphere-derived cells were cultured for 3 days in FGF-2/EGF-free medium with or without screening samples, and the cell proliferation rate was then determined using the Alamar Blue assay. Among the compounds from the medicinal herbs tested, FA (Fig. 1A) had the strongest promoting activity for NSC/NPC proliferation and exhibited a dose-dependent effect (Fig. 1B). In contrast, other cinnamic acid derivatives such as 3-methoxycinnamic acid, 3-hydroxycinnamic acid, 3,4-dimethoxycinnamic acid, 3-hydroxy-4-methoxycinnamic acid, 2,3-dimethoxycinnamic acid, 2,4-dimethoxycinnamic acid, 2,5-dimethoxycinnamic acid, and 4-hydroxycinnamic acid had no effect (Table 1). Since FA has a strong anti-oxidant activity, we examined the effect of other anti-oxidants (ascorbic acid, baicalein and epigallocatechin gallate), which have different chemical structures, on the proliferation of cultured NSC/NPCs. The anti-oxidants except for FA did not affect the proliferation of NSC/NPCs in the concentration range of 0.1 to 100 μg/ml (Fig. 1C and our unpublished data). We next examined the effect of FA on cell proliferation using a BrdU-incorporation assay. Since BrdU, an analogue of thymidine, is incorporated into dividing and proliferating cells, BrdU labeling assay has been widely used in many investigations concerning NSC/NPCs proliferation. NSC/NSCs were incubated with or without FA (10 μg/ml) for 5 h and were subsequently incubated with BrdU (5 μM) for 2 h. BrdU-incorpolated cells were detected by immunocytochemistry with anti-BrdU antibody. As shown in Fig. 1D, the percentage of BrdU-labeled cells was significantly increased by FA treatment. To provide further evidence that FA promotes NSC/NPC proliferation, a neurosphere formation assay was performed. FA alone was not able to sustain neurosphere formation in the absence of FGF-2/EGF (Fig. 1E). However, when the cells were plated in growth medium containing FGF-2 and EGF, the number (Fig. 1E) and size (Fig. 1F) of neurospheres increased by addition of 10 μg/ml of FA.

Effect of FA on differentiation of cultured NSC/NPCs

We next examined whether FA affects differentiation of NSC/NPCs. In agreement with the results of the *in vitro* proliferation assay (Fig. 1), the total cell number was significantly increased in FA-treated cultures (Fig. 2A and 2B left panel). Although the numbers of Tuj-1-positive cells (neurons) and GFAP-positive cells (astrocytes) also increased in FA-treated cultures (Fig. 2B middle and right panel), FA did not affect the ratio of either Tuj1-positive cells (Fig. 2C left) or GFAP-positive cells (Fig. 2C right) in the total cell population. These observations suggest that the FA-induced number of Tuj-1-positive neurons is based
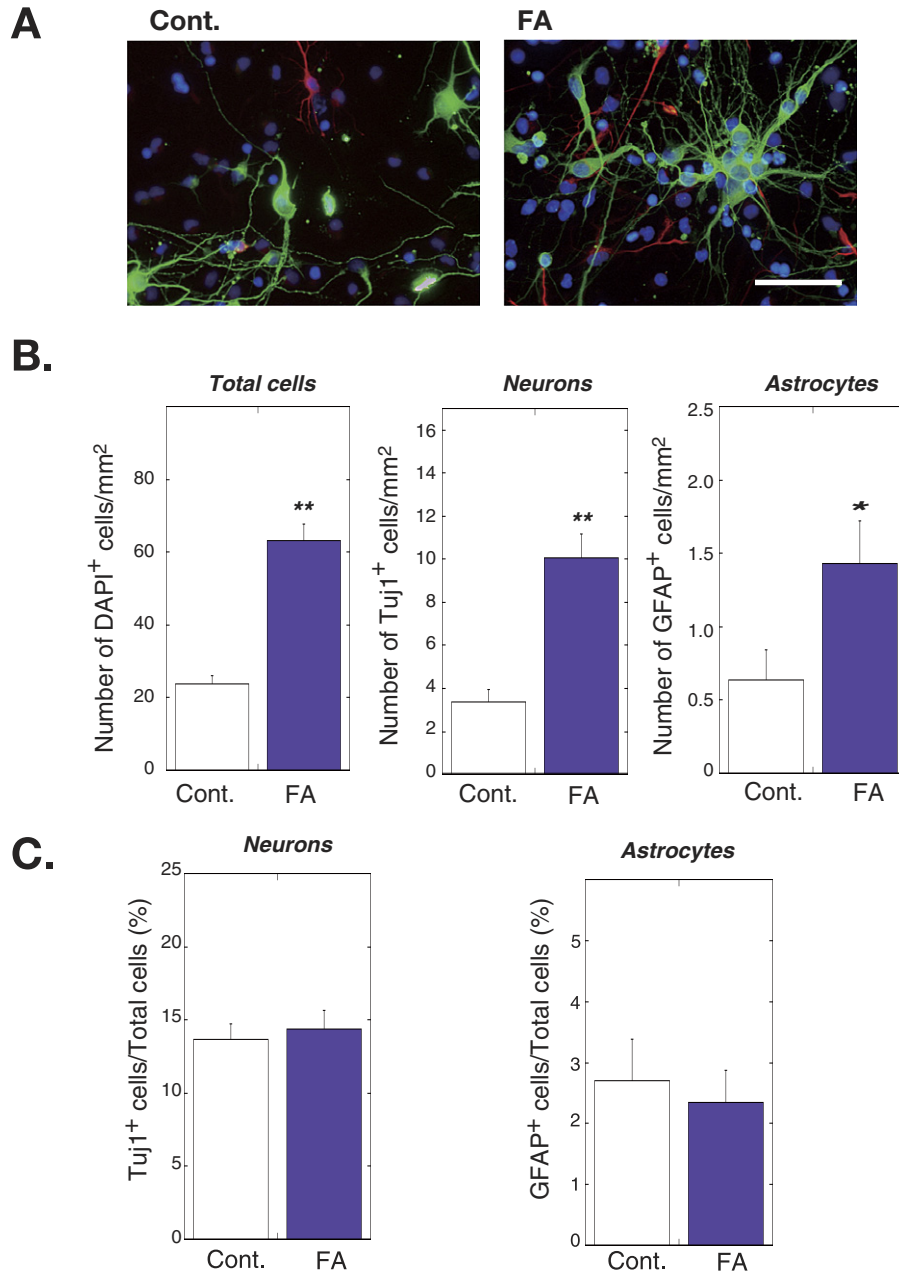
Fig. 2. FA does not affect the differentiation of NSC/NPCs. (A) Representative immunofluorescence images of FA-treated NSC/NPCs. Dissociated NSC/NPCs were cultured on laminin/poly-L-ornithine-coated 8-well chamber slides with FA (10 μg/ml) for 5 d. Cells were fixed and stained for Tuj-1 (green, marker for immature neurons), GFAP (red; marker for astrocytes) and DAPI (blue; nuclear). Scale bar=50 μm. (B) Number of total cells (left panel), Tuj-1-positive cells (center panel) and GFAP-positive cells (right panel). Data shown represent mean±SE of 10 fields selected randomly from eight-well chamber slides. * $P<0.05$, ** $P<0.0001$. Similar results were obtained in three independent experiments. (C) Ratio of Tuj-1-positive cells (left panel) and GFAP-positive cells (right panel). Data shown represent the mean±SE of 10 fields selected randomly from eight-well chamber slides. Similar results were obtained in three independent experiments.

on NSC/NPC proliferation rather than regulated cell differentiation.

Oral administration of FA increases the number of BrdU-positive cells in the DG of CORT-treated mice

In order to evaluate whether FA is capable of regulating adult NSC/NPC proliferation *in vivo*, we utilized CORT-treated mice (Fig. 3A). BrdU (100 mg/kg) was injected intraperitoneally at day 6 and 8. The dividing cells labeled with BrdU were visualized using BrdU immunohistochemistry. BrdU-labelled neurons were found in all treatment groups in the dentate gyrus of the hippocampus (Fig. 3C). CORT significantly decreased the number of BrdU-positive cells in the DG compared with the vehicle-treated group ($P<0.05$; Fig. 3B and 3C middle).
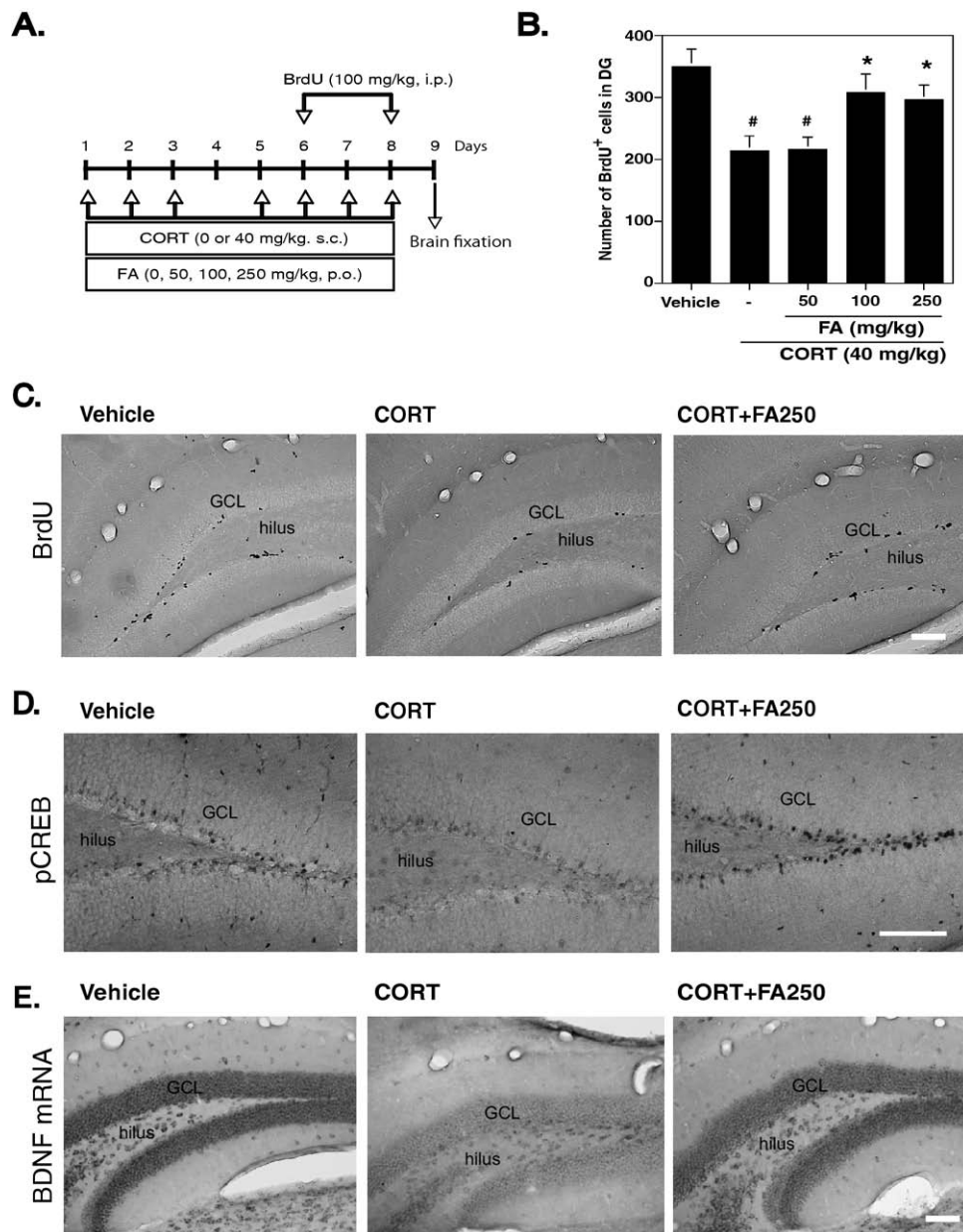
Fig. 3. FA induces the proliferation of NSC/NPCs in the DG of corticosterone (CORT)-treated mice. (A) Schematic representation of the experimental design. A daily subcutaneously injection of CORT (40 mg/kg) in sesame oil or oil alone was given for 8 d. FA (50, 100 or 250 mg/kg) was administered orally for the same period of CORT administration. BrdU (100 mg/kg) was i.p. injected at day 6 and 8. (B) Number of BrdU-positive cells in the DG. Each column represents the mean±SEM (n=10). # P<0.005 versus the vehicle-treated group. * P<0.05 versus the CORT-treated group. Scale bar=100 μm. (C) Representative photomicrographs showing BrdU-positive cells in the DG of vehicle- (left panel), CORT- (middle panel) and CORT+FA250-treated (right panel; 250 mg FA/kg/d) mice. (D) Representative photomicrographs showing pCREB-positive cells in the DG of vehicle- (left), CORT- (middle), and CORT+FA-treated (right; 250 mg FA/kg/d) mice (n=10). Scale bar=100 μm. (E) Representative photomicrographs showing BDNF mRNA expression in the DG of vehicle- (left), CORT- (middle), and CORT+FA-treated (right; 250 mg FA/kg/d) mice (n=10). The expression pattern of BDNF mRNA in the DG was detected using *in situ* hybridization with the antisense cRNA probe to mouse BDNF mRNA. Scale bar=100 μm.

FA (100 or 250 mg/kg) prevented the suppressive effect of CORT on hippocampal cell proliferation in adult mice (P<0.05; Fig. 3B and 3C). When FA (250 mg/kg) was administered to intact mice (without CORT), the number of BrdU positive cells was not influenced by FA (Supplementary Fig. 1), suggesting that FA restored CORT-induced decrease of hippocampal cells proliferation.

Effects of FA on CREB phosphorylation and BDNF mRNA expression

Several studies have shown that cAMP response element binding protein (CREB) signaling enhances hippocampal neurogenesis (Nibuya et al., 1996; Nakagawa et al., 2002). Therefore, immunostaining of phosphorylated CREB (pCREB),
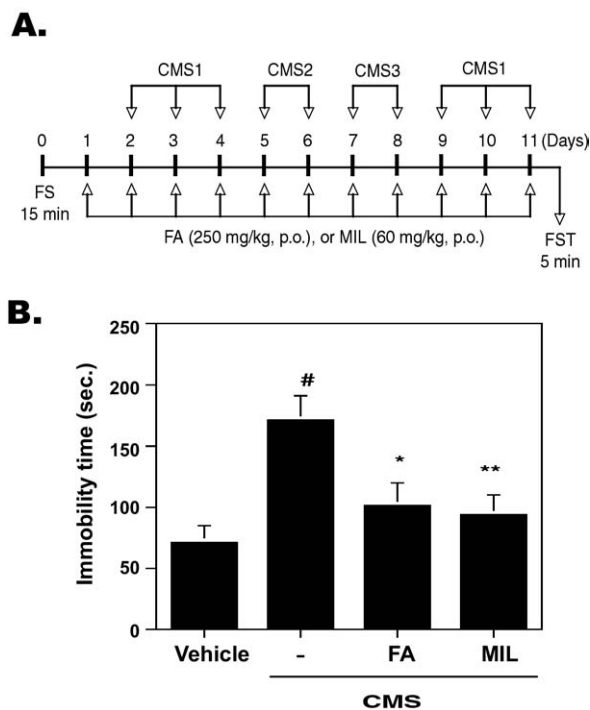
A.

B.

Fig. 4. FA ameliorates CMS-induced depression-like behavior. (A) Schematic representation of the experimental design for the behavioral study. Mice were placed individually into water for 15 min (FS). After 2 d, the mice were exposed to chronic mild stress consisting of three different stress situations (CMS 1, CMS 2 and CMS 3). The mice were then placed again into water at 60 min after the final treatment of the drug, and the total duration of immobility was measured during a 5 min forced swimming test (FST). Drugs were administered from day 1 to day 11 (11 times). (B) One hour after the last administration of FA (250 mg/kg/d) or milnacipran (MIL; 60 mg/kg/d), the immobility time was measured during 5 min of forced swimming. Each column represents the mean±SEM of 12 mice per group. # $P<0.0005$ versus vehicle. * $P<0.01$, ** $P<0.005$ versus CMS-applied group.

which is an activated form of CREB, was performed. As shown in Fig. 3D, pCREB immunostaining was mainly localized in a region close to or in the SGZ of the hippocampus. Administration of CORT (250 mg/kg) resulted in the reduction of the intensity of pCREB immunostaining, whereas the number of pCREB positive cells in DG was not changed by CORT treatment (Fig. 3D middle panel). FA administration strongly enhanced the intensity of pCREB immunostaining in DG compared with vehicle- and CORT-treated animals (Fig. 3D right panel), but the number of pCREB positive cells was not changed among the groups.

Since BDNF is also known as an intrinsic modulator of neurogenesis, we employed in situ hybridization to examine the effect of FA on BDNF mRNA expression in the DG of CORT-treated mice. The expression of BDNF mRNA was mainly detected in the granular cell layer (GCL) of DG (Fig. 3E). Although BDNF mRNA expression decreased throughout the GCL of the DG in CORT-treated mice (Fig. 3E middle panel), FA-treatment attenuated CORT-induced downregulation of BDNF mRNA expression (Fig. 3E right panel).

FA ameliorates stress-induced depression-like behavior

In order to evaluate whether FA has anti-depression-like activity, we utilized depression-like model mice, which were prepared by a combination of FS with three different combined CMS (Fig. 4A). As previously reported (Ito et al., 2006), stress caused by the combination of FS and CMS resulted in a significant prolongation of the immobility time in the FST (Fig. 4B). Oral administration of FA significantly reduced the duration of immobility compared to water-administered stress-applied mice ($P<0.001$, Fig. 4B). Milnacipran, an inhibitor of both norepinephrine and serotonin transporters (SNRI), also significantly reduced the duration of immobility ($P<0.005$, Fig. 4B). Both FA and milnacipran did not affect spontaneous locomotor activities in the open field test (data not shown).

DISCUSSION

FA is a plant constituent that is often contained in several medicinal herbs. Although the pharmacological effects of FA have been reported by many researchers (Srinivasan et al., 2007), the effects of FA on NSC/NPCs have not yet been addressed. In the present study, we have shown that FA promoted the proliferation of cultured NSC/NPCs in a dose-dependent manner (Fig. 1B, C, D), and also increased the number and size of secondary generated neurospheres (Fig. 1E, F). Since neurospheres contain both NSCs and NPCs, it is still unclear which types of cells were proliferated by FA under these conditions. However, FA alone was not able to sustain neurosphere formation in the absence of growth factors (Fig. 1E), suggesting that FA might promote the proliferation of lineage-restricted cells such as NPCs rather than NSCs. Since FA has strong antioxidant activity, we also tested whether other antioxidants affect the proliferation of NSC/NPCs. Application of antioxidants such as ascorbic acid, baicalein and epigallocatechin gallate did not affect the proliferation of cultured NSC/NPCs (Fig. 1C). Furthermore, cinnamic acid

Table 1. Effect of cinnamic acid derivatives on cultured NSC/NPC proliferation

Compound	Proliferation rate (% of control)
Control	100.0±16.0
FA (4-Hydroxy-3-methoxycinnamic acid)	256.4±14.4*
3-Methoxycinnamic acid	109.3±15.4
3-Hydroxycinnamic acid	118.6±17.2
3,4-Dimethoxycinnamic acid	126.8±18.4
3-Hydroxy-4-methoxycinnamic acid	131.1±19.5
2,3-Dimethoxycinnamic acid	132.3±25.2
2,4-Dimethoxycinnamic acid	113.2±11.1
2,5-Dimethoxycinnamic acid	116.2±12.8
4-Hydroxycinnamic acid	116.2±15.7

Dissociated NSC/NPCs were cultured on a poly-L-lysine-coated 96-well plate with cinnamic acid derivatives (10 μg/ml) for 3 days. Cell proliferation was measured using an Alamar Blue assay.
* $P<0.01$ versus control. Data shown represent the mean±SD ($n=3$). Similar results were obtained in three independent experiments.

derivatives except for FA did not exhibit the stimulation effect of NSC/NPC proliferation in culture (Table 1). These observations suggest that the promotion of NSC/NPC proliferation is not a common feature of antioxidants and cinnamic acid derivatives. Since FA has a protective effect for some neuronal populations (Kanski et al., 2002; Antony et al., 2004; Sultana et al., 2005), we set out to determine whether survival of NSC/NPCs increased in FA-treated cultures. The results showed that the survival rate was not changed by FA treatment (data not shown). To examine the effect of FA on differentiation of NSC/NPCs, we conducted a fluorescence immunohistochemical analysis with neuronal marker Tuj-1 and astrocyte marker GFAP. Although the total number of Tuj1- and GFAP-positive cells increased significantly by FA treatment, the ratio of both Tuj-1-positive cells and GFAP-positive cells did not change (Fig. 2). These observations suggest that increase of the neuronal population by FA is a result of NSC/NPC proliferation, but not differentiation.

In order to examine the *in vivo* effects of FA on proliferation of NSC/NPCs, CORT-administered mice and BrdU labeling were employed. CORT is a principal glucocorticoid synthesized in the rodent adrenal cortex and secreted in response to stress (Hyde and Skelton, 1961). The hippocampus is a vulnerable region prone to damage by application of stress or CORT. In animal experiments, the stress-induced increase of CORT secretion or exogenous application of a high dose of CORT elicit neuronal atrophy in the hippocampal subfield CA3 and decrease proliferation of NPCs in the dentate gyrus (Watanabe et al., 1992; Cameron and Gould, 1994; Magarinos and McEwen, 1995; Gould et al., 1997). In accordance with previous reports, repeated administration of CORT resulted in a decreased number of BrdU-positive cells in the dentate gyrus compared with vehicle-treated mice (Fig. 3B, C). However, FA treatment significantly increased the CORT-induced decrease in the number of BrdU-positive cells in the dentate gyrus, suggesting that oral administration of FA for 8 days increases NSC/NPC proliferation.

As the FST is commonly employed as a behavioral screen for antidepressant treatments (Porsolt et al., 1977; Thiebot et al., 1992), we used this test to estimate the antidepressant-like effect of FA in this study. Consistent with previous reports, CMS exposure resulted in a prolongation of the immobility time in the FST (Fig. 4B), suggesting that CMS-treated mice exhibited depression-like behavior. Meanwhile, FA treatment significantly reduced the immobility time of CMS-exposed mice, suggesting that FA produces an antidepressant-like effect. The precise mechanism by which FA ameliorates CMS-induced depression-like behavior remains unclear, although the modulation of neurogenesis by FA might be involved in this ameliorative effect.

Several papers have reported that chronic stress exposure and/or repeated CORT administration decreases hippocampal neurogenesis, CREB phosphorylation and BDNF expression (Schaaf et al., 1997; Gould and Tanapat, 1999; Luo et al., 2005). In agreement with previous studies, repeated CORT administration in the present study downregulated hippocampal cell proliferation (Fig. 3B, C), the phosphorylation of CREB (Fig. 3D), and the expression of BDNF mRNA (Fig. 3E). CRE has been implicated in the regulation of the expression of many genes and cellular processes important in brain function (Lonze and Ginty, 2002). A constitutive binding of CREB to CRE consensus sequences in the absence of a stimulus has been reported (Quinn and Granner, 1990), where phosphorylation of CREB results in the enhancement of transcriptional activation by the recruitment of additional coactivators (Chrivia et al., 1993; Quinn, 1993). Nakagawa et al. (2002) reported that activation of the cAMP-CREB cascade by administration of rolipram, an inhibitor of cAMP breakdown, increased the proliferation of hippocampal newborn cells, and that the increased cell proliferation by rolipram was attenuated by overexpression of dominant negative mutant CREB. Chronic administration of various antidepressants increases phosphorylation of CREB and its downstream target gene BDNF in the hippocampus (Nibuya et al., 1996; Thome et al., 2000). Furthermore, administration of BDNF ameliorates CORT-induced depression-like behavior (Gourley et al., 2008). In this paper, oral administration of FA increased CREB phosphorylation and BDNF mRNA expression of the hippocampus in CORT-treated mice (Fig. 3D, E), suggesting that the induction of these factors might be involved in the regulation of hippocampal progenitor cells and amelioration of CORT-induced depression-like behavior by FA. Since several reports have demonstrated that CREB and/or BDNF can regulate NSC/NPC survival, we cannot exclude the possibility that FA modulates not only hippocampal cell proliferation, but also survival of hippocampal cells in CORT-treated mice. Further studies are needed to clarify the functional significance of the induction of pCREB and BDNF by FA.

CONCLUSION

We have provided the first evidence that FA increases the proliferation of NSCs/NPCs *in vitro* and *in vivo*, and ameliorates stress-induced depression-like behavior in mice. These novel pharmacological effects of FA may be useful for the treatment of mood disorders such as depression.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroscience.2009.10.023.