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4′-Demethylnobiletin, a Bioactive Metabolite of Nobiletin Enhancing PKA/ERK/CREB Signaling, Rescues Learning Impairment Associated with NMDA Receptor Antagonism via Stimulation of the ERK Cascade†

Md. Al Rahim,‡ Akira Nakajima,‡ Daisuke Saigusa,‡,§ Naomi Tetsu,‡ Yuji Maruyama,‡ Masatoshi Shibuya,∥ Hiroyuki Yamakoshi,∥ Yoshihisa Tomioka,§ Yoshiharu Iwabuchi,∥ Yasushi Ohizumi,⊥,@,# and Tohru Yamakuni*,‡

‡*Laboratory of Pharmacotherapy, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan,* §*Laboratory of Oncology, Pharmacy Practice and Sciences, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan,* ∥*Department of Synthetic Chemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan,* ⊥*Yokohama College of Pharmacy, 601 Matano-cho, Tozuka-ku, Yokohama 245-0066, Japan,* @*Research Center of Supercritical Fluid Technology, Graduate School of Engineering, Tohoku University, 6-6-11-404 Aoba, Aramaki, Aoba-ku, Sendai 980-8579, Japan, and* #*Department of Pharmacokinetics and Pharmacodynamics, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan*

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ABSTRACT: The biochemical and pharmacological activities of nobiletin, including neurotrophic and memory-enhancing action, in both in vitro and in vivo systems are well established. However, whether its metabolites do have such beneficial effects like nobiletin remains to be examined. Here we, for the first time, report that 2-(4-hydroxy-3-methoxyphenyl)-5,6,7,8-tetramethoxychromen-4-one (4′-demethylnobiletin), a major metabolite of nobiletin identified in the urine of rats and mice, stimulates the phosphorylation of ERK and CREB and enhances CRE-mediated transcription by activating a PKA/MEK/ERK pathway, like nobiletin, in cultured hippocampal neurons. Since NMDA receptor-mediated ERK signaling is involved in memory processing, including associative memories, we also examined whether 4′-demethylnobiletin, by activating ERK signaling, could restore learning impairment. Chronic intraperitoneal (ip) treatment of the mice with 10 or 50 mg of 4′-demethylnobiletin/kg rescued the NMDA receptor antagonist MK-801-induced learning impairment, accompanied by improvement of the MK-801-induced decrease in the level of ERK phosphorylation in the hippocampus of the animals. Consistently, 4′-demethylnobiletin also restored MK-801-induced inhibition of NMDA-stimulated phosphorylation of not only ERK but also PKA substrates in cultured rat hippocampal neurons. Moreover, we actually detected 4′-demethylnobiletin in the brain of mice following acute ip administration, demonstrating that the metabolite can cross the blood−brain barrier to reach the brain and thereby exert its effects to reverse learning impairment. Therefore, these results suggest that 4′-demethylnobiletin, a bioactive metabolite of nobiletin, may serve as a potential therapeutic agent, at least, for memory disorders associated with a dysregulated NMDA receptor ERK signaling, like nobiletin.

Cyclic AMP-dependent protein kinase (PKA)[1] has been strongly implicated in hippocampal long-term potentiation (LTP) associated with learning and memory (*1*, *2*). Together, a large body of evidence consolidates the notion that impaired signaling through the cAMP/PKA pathway has been observed in a variety of disease processes, including age-related neurodegenerative diseases such as Alzheimer's disease (AD) (*3*). Also, the focus of attention is signaling via mitogen-activated protein kinase (MAPK) or ERK (*4*), because stimulation of the ERK signaling pathway is important for memory formation (*5*−*7*). Notably, cross talk also exists between cAMP/PKA and ERK signaling pathways in PC12 cells (*8*), hippocampal neurons in culture (*9*), and acute hippocampal slices (*10*); an increase in the intracellular concentration of cAMP induces stimulation of PKA activity and thereby activates MAPK/ERK kinase (MEK), through Rap1/B-Raf, which in turn enhances ERK activity. Furthermore, cAMP/PKA and ERK signaling pathways couple with cAMP response element binding protein (CREB)/CRE-mediated transcription downstream. Accordingly, the CREB/CRE-mediated transcriptional pathway is pivotal for synaptic plasticity (*11*).

N-Methyl-D-aspartate (NMDA) receptors, which are widely distributed in the brain with the highest level in the CA1 region of hippocampus, have been placed in the center of the learning process (*12*). Activation of the NMDA receptor population is necessary for fear conditioning learning, a robust form of associative memory task (*6*). Stimulation of NMDA receptors

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*To whom correspondence should be addressed. Telephone: +81-22-795-6853. Fax: +81-22-795-6850. E-mail: yamakuni@mail.pharm.tohoku.ac.jp.

[1]Abbreviations: AD, Alzheimer's disease; CREB, cAMP response element binding protein; ERK, extracellular signal-regulated kinase; LTP, long-term potentiation; MEK, mitogen-activated protein kinase kinase; NMDA, *N*-methyl-D-aspartate; PDE, phosphodiesterase; PKA, cyclic AMP-dependent protein kinase; SEM, standard error of the mean.

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leads to activation of a series of events, including the cAMP-dependent signaling pathway, that eventually converge upon ERK (*13*). Accordingly, recent studies have demonstrated that learning-associated activation of ERK via NMDA receptors is necessary for consolidation of the resultant learning (*6*, *14*, *15*). Consistent with these observations, blockade of NMDA receptors or ERK signaling in vivo leads to impairment of associative learning (*6*, *16*). Taken together, it has been suggested that the regulation of ERK signaling via the NMDA receptor is a potential critical step in clinical control of cognitive dysfunction. Therefore, these observations raise the possibility that agents which rescue NMDA receptor antagonist MK-801-induced inhibition of learning-elicited activation of ERK could be of value in the treatment of cognitive dysfunction associated with a hypofunction of NMDA receptor-mediated ERK signaling.

Recently, we have successfully identified nobiletin, a citrus polymethoxyflavone, as a potential candidate for the development of a drug for cognitive dysfunction. Nobiletin, which has the ability to augment PKA-mediated phosphorylation of GluR1 and the postsynaptic receptor response to glutamate in murine hippocampus (*17*), rescues memory impairments in AD model rats (*18*), mice associated with NMDA receptor antagonism (*19*), olfactory-bulbectomized mice exhibiting cholinergic neurodegeneration (*20*), and an amyloid precursor protein (APP) transgenic mouse line as well (*21*). It is also noteworthy that nobiletin is metabolized to demethylated derivatives, and the major urinary metabolite is demethylated at the C-4′ position in rats and mice (*22*, *23*). In addition, nobiletin is biotransformed to 4′-demethylated metabolite by *Aspergillus niger*, a fungus (*24*). Thus, we examined whether the in vitro and in vivo effects of a major metabolite of nobiletin were comparable to those of nobiletin. In this study, we present the first evidence that 4′-demethylnobiletin, a major in vivo metabolite of nobiletin, not only activates PKA/ERK/CREB signaling to enhance CRE-mediated transcription in cultured hippocampal neurons but also reaches the brain to rescue memory impairment associated with NMDA receptor antagonism by activation of ERK signaling in mice.

MATERIALS AND METHODS

Reagents. HPLC-grade acetonitrile (CH_3CN) and methanol (MeOH) from Kanto chemical and formic acid (HCOOH) from Wako Pure Chemical industries were used as organic solvents. Tween 80 and sodium pentobarbital were obtained from Kanto chemical and Dainippon (Osaka, Japan), respectively. Bond Elut C18 (500 mg, 3 mL) was purchased from Varian (Palo Alto, CA).

Synthesis of 4′-Demethylnobiletin. Through a series of chemical reactions described in the Supporting Information (see also Figure 2 of the Supporting Information), 4′-demethylnobiletin was obtained as the final product.

Animals. Male ddY mice (8 weeks old) used for both behavioral experiments and the detection of 4′-demethylnobiletin by HPLC/UV systems were obtained from Nippon SLC (Hamamatsu, Japan). Animals were housed in cages with free access to food and water at a constant temperature (23 ± 1 °C) and humidity (55±5%) and adapted to a standard 12 h light−12 h dark cycle (light cycle from 9:00 to 21:00). All procedures used in these in vitro and in vivo studies were approved by the Committee on the Care and Use of Experimental Animals, Tohoku University, in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Hippocampal Neuronal Culture and Treatment. Primary cultures of hippocampal neurons were prepared as described previously with minor modifications (*18*, *25*). In brief, under ether anesthesia of an 18-day-old Sprague-Dawley (SD) rat (Japan, SLC), embryos were immediately decapitated. The brain was quickly removed, and hippocampus was collected in ice-cold Dulbecco's phosphate-buffered saline under sterile conditions. The hippocampal neurons were dissociated according to the standard methods with SUMITOMO Nerve-Cell Culture System (SUMITOMO BAKELITE), plated on a 35 mm culture dish coated with poly-L-lysine (Life Technologies) at a density of 1×10^6 cells/dish in neurobasal medium without phenol red (Gibco Co., Ltd.) containing B-27 supplement (Gibco Co., Ltd.), 500 μM L-glutamine, and 0.005% penicillin-streptomycin, and cultured at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air. At 4 days in vitro (DIV), half of the medium was replaced with the medium containing a mitotic inhibitor, cytosine arabinofuranoside (Ara-C), to inhibit non-neuronal proliferation.

At 10−14 DIV, cultured hippocampal neurons were treated with 4′-demethylnobiletin, vehicle (0.1% DMSO), or other agents as appropriate, in the Ara-C-containing medium without B-27 supplement and L-glutamine at the concentrations and time points indicated in the figure legends, and thereafter subjected to Western blot analysis.

Contextual Fear Conditioning Experiment. Animals were placed in the training chamber and allowed to explore for 2 min, after which they received an electric shock (2 s, 0.7 mA). The 2 min/2 s shock paradigm was repeated for a total of three shocks. After the last shock, animals were allowed to explore the context for an additional 1 min prior to being removed from the training chamber. During training, freezing behavior was measured during 1 min after each shock. To assess contextual learning and memory, the animals were placed back into the training context 24 h after fear conditioning and scored for freezing for 5 min. Freezing behavior was assessed by observing the animals every 5 s. Vehicle (0.5% Tween 80) or 4′-demethylnobiletin [10 or 50 mg/kg, intraperitoneally (ip)] was injected once daily for seven consecutive days (days 1−7). On day 7, vehicle or 4′-demethylnobiletin (10 or 50 mg/kg) was injected 10 min prior to saline or MK-801 [0.08 mg/kg, subcutaneously (sc)], which was given 30 min before training with the contextual fear conditioning paradigm. The dose used for 4′-demethylnobiletin was same as that of nobiletin described previously (*19*), and the injection times were chosen on the basis of our previous study showing that repeated treatment with nobiletin reversed MK-801-induced learning impairment by 50−90%, whereas a single treatment with nobiletin led to a moderate effect (*19*). The dose of MK-801 was chosen on the basis of a previous study showing that MK-801 (0.08 mg/kg) inhibited both contextual associative learning and activation of ERK during fear conditioning (*6*).

Open-Field Test. Vehicle or 4′-demethylnobiletin (10 or 50 mg/kg, ip) was injected once daily for seven consecutive days (days 1−7). Twenty-four hours after the last injection, mice were placed into the corner of an open-field box (50 cm × 50 cm × 45 cm) and allowed to freely explore for 5 min. Horizontal activities of mice were measured using a computer-assisted video tracking system (CompACT vas, Muromachi Kikai, Tokyo, Japan).

Mice Brain Sample Preparation for the Assessment of 4′-Demethylnobiletin by the High-Performance Liquid Chromatography/Ultraviolet Method (HPLC/UV). To address the question of whether 4′-demethylnobiletin crosses the

blood−brain barrier to reach the brain, 4′-demethylnobiletin (50 mg/kg, suspended in a 0.5% Tween 80 solution) was ip injected into male ddY mice. Mice were then deeply anesthetized with sodium pentobarbital. Then, 3, 10, and 30 min after administration of 4′-demethylnobiletin, mice were rapidly perfused transcardially with cold saline through the left ventricle and thereby sacrificed. Subsequently, the brains were quickly removed, pulverized, and stored at −80 °C. These brain samples were subjected to determination of the amount of 4′-demethylnobiletin by the HPLC/UV method described below.

Sample Preparation for HPLC/UV. Sample preparation for HPLC/UV was performed as follows. Approximately 50 mg of each pulverized brain sample was placed into a 2 mL sample tube. Then, 1 mL of solution (MeOH/H_2O, 70/30, v/v) and 50 μL (1 ng/μL) of an IS [5,6,7,8-tetramethoxy-2-(4-nitrophenyl)chromen-4-one] were added. This mixture was further homogenized for 30 min by ultrasonication. After centrifugation at 15000*g* for 20 min at 4 °C, the supernatant was collected in a 10 mL glass tube and diluted by addition of 2 mL of H_2O. The diluted sample was then applied to Bond Elut C18 conditioned with 2 mL of MeOH and 2 mL of H_2O. The cartridge was washed with 3 mL of H_2O and 3 mL of the organic solutions (MeOH/H_2O, 50/50, v/v). Then, 4′-demethylnobiletin was eluted with 7 mL of the organic solution (MeOH/H_2O, 80/20, v/v), and the eluate was dried under a nitrogen stream. Finally, the dried residue was dissolved with 50 μL of the solution (CH_3CN/H_2O, 50/50, v/v), mixed for 30 s, and passed through the YMC Duo-Filter (QDUO 04, pore size of 0.2 μm). The filtered sample was retained for the subsequent HPLC/UV determination.

Assessment of 4′-Demethylnobiletin via HPLC/UV. The method for detection of 4′-demethylnobiletin via HPLC/UV will be described in detail elsewhere (D. Saigusa et al., manuscript in preparation). Briefly, the column for this study was a Luna C18, 5 μm model (150 mm × 2.1 mm, Phenomenex) maintained at 35 °C. The NANOSPACE SI-2 LC system was used, comprising a LC pump, an autosampler and online degasser (Shiseido), and a variable-wavelength UV detector set to 335 nm. Gradient elution was performed using 0.1% formic acid (eluent A) and CH_3CN (eluent B). The gradient elution initial conditions were 35% B with a linear gradient to 55% from 4 to 6 min, followed by a linear gradient to 70% B at 10 min, this proportion being maintained for 8 min. The mobile phase was then returned to the initial conditions immediately after 18 min and maintained until the end of the run at 20 min. The flow rate was 200 μL/min, and the injection volume was 1 μL.

Western Blot Analysis. Western blot analyses of cell extracts from hippocampal neuronal cultures were conducted, as described previously (*18*, *25*). After treatment with 4′-demethylnobiletin, or other agents as appropriate, cells were lysed with 60 μL of lysis buffer [1 mM EDTA, 1% SDS, 10 mM NaF, 10 nM calyculin, 320 nM okadaic acid, 1 μM sodium orthovanadate, 1 mM *p*-APMSF, 10 μg/mL pepstatin, 10 μg/mL antipain, 10 μg/mL leupeptin, 10 μg/mL chymostatin, 10 μg/mL phosphoramidon, and 10 mM HEPES (pH 7.5)] per 35 mm dish. Cell lysates were heated at 95 °C for 5 min, sonicated, and centrifuged at 14000*g* for 20 min at 4 °C to yield the supernatant as cell extracts. Cell extracts were separated by 12.5% SDS−PAGE and transferred onto a PVDF membrane. The membranes were incubated with anti-phospho-PKA substrate, anti-phospho-ERK (Thr202/Tyr204), anti-total ERK, anti-PKAα, anti-phospho-CREB (Ser133), and anti-total CREB antibodies (1:1000, Cell Signaling Technology). Immunoreactive proteins were visualized by chemiluminescence, as described previously (*18*). The band intensities were quantitatively analyzed using SCION.

Western blot analysis of mouse brain hippocampus was performed as described previously (*19*) with a minor modification. In brief, mice were treated with 4′-demethylnobiletin (50 mg/kg) once daily for seven consecutive days (i.e., days 1−7). On day 7, 4′-demethylnobiletin was administered 10 min prior to MK-801 (0.08 mg/kg, sc), which was given 30 min before the training trial of the fear conditioning task. Sixty minutes after being trained, mice were killed by decapitation, and the brains were immediately removed. Hippocampi were homogenized in ice-cold homogenization buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1% NP-40, 0.5 mM DTT, 1 mM *p*-APMSF, 1 mM sodium orthovanadate, 10 mM NaF, 4 μg/mL pepstatin, 4 μg/mL leupeptin, 4 μg/mL antipain, and 4 μg/mL chymostatin]. The homogenate was centrifuged at 12000*g* at 4 °C for 10 min. The supernatant was collected, supplemented with sample buffer, and boiled at 95 °C for 5 min. The prepared sample was thus ready for Western blot experiments. An equal amount of protein (20 μg) was subjected to SDS−polyacrylamide gel electrophoresis (12.5% gels), and the blotted membrane was blocked in TBST buffer containing 5% skim milk for 2 h at room temperature. The membrane was then incubated with anti-phospho-ERK (Thr 202/Tyr 204) antibody. Following stripping of the antibody, the membrane was reprobed with anti-ERK antibody. To evaluate the ERK activation, the phospho-ERK levels were normalized to the total ERK levels in the same membranes.

Reporter Gene Assay. Neuronal cells were plated on 48-well plates at a density of 8×10^4 cells/well and cultured for 10−14 days. Transfection and reporter gene assays were conducted as described previously (*25*). A firefly luciferase reporter plasmid containing CRE inserted into the upstream of a TATA-like promoter (pTAL) region taken from herpes simplex thymidine kinase promoter was purchased from Clontech. A Renilla luciferase control vector, pRG-TK (Promega), was also used as an internal control to normalize the difference in transfection efficiency. Cultured neuronal cells were transfected by lipofection using lipofectAMINE 2000 (Invitrogen).

Statistical Analyses. One-way ANOVA, followed by post hoc correction according to Tukey, was performed. The data for the freezing during training of contextual fear conditioning paradigm were analyzed by two-way repeated measures ANOVA. A $P < 0.05$ level was considered to be statistically significant.

RESULTS

4′-Demethylnobiletin Time- and Concentration-Dependently Activates Phosphorylation of ERK and CREB in a PKA/MEK/ERK Pathway-Dependent Manner in Cultured Hippocampal Neurons. Nobiletin time- and concentration-dependently increases the level of phosphorylation of ERK and one of its downstream effectors, CREB, in hippocampal neurons (*25*, *26*). Like nobiletin, its major urinary metabolite in rats, 4′-demethylnobiletin (Figure 1) induced a persistent increase in the level of phosphorylation of ERK, accompanied by a sustained augmentation of phosphorylation of CREB in hippocampal neurons [P-ERK, $F_{(6,21)} = 3.4394$, $p = 0.0159$ by one-way ANOVA; P-CREB, $F_{(6,14)} = 3.8464$, $p = 0.0177$ by one-way ANOVA]. The phosphorylation level of ERK and CREB reached a maximum 5 min after treatment with this compound at 30 μM, and the maximum level persisted until 10 min after

treatment. The persistent effect on phosphorylation of ERK and CREB was observed for 60 min, as treated with 4′-demethylnobiletin (Figure 2A). Thus, treatment with 30 μM 4′-demethylnobiletin, for either 5 or 10 min, could yield equipotent effects on the stimulation of phosphorylation of ERK and CREB. The concentration-dependent effects of 4′-demethylnobiletin were also tested at the concentrations ranging from 1 to 100 μM for 10 min. The phosphorylation of ERK and CREB was observed to increase in cultured hippocampal neurons after treatment with the compound at concentrations of 10−100 μM (Figure 2B), indicating the concentration-dependent effects on phosphorylation of ERK and CREB in cultured hippocampal neurons [P-ERK, $F_{(6,14)} = 5.0388$, $p = 0.006$ by one-way ANOVA; P-CREB, $F_{(6,14)} = 5.0799$, $p = 0.0058$ by one-way ANOVA].

We have previously found that nobiletin activates a cAMP/PKA/MEK-dependent signaling to enhance the phosphorylation of ERK and CREB (*18*, *25*). As shown in Figure 1 of the Supporting Information, 30 μM 4′-demethylnobiletin appreciably enhanced the phosphorylation of PKA substrates. To assess the effects of protein kinase inhibitors on the 4′-demethylnobiletin-induced intracellular signaling, we employed H-89, a PKA inhibitor, and U0126, a MEK inhibitor. Our study revealed that the 4′-demethylnobiletin-induced increase in the level of phosphorylation of ERK and CREB was almost abolished by 10 μM with both H-89 and U0126, suggesting the involvement of a PKA/MEK-dependent signaling in the stimulation of ERK and CREB phosphorylation in cultured hippocampal neurons [P-ERK, $F_{(3,8)} = 12.582$, $p = 0.0021$ by one-way ANOVA; P-CREB, $F_{(3,8)} = 9.7529$, $p = 0.0048$ by one-way ANOVA] (Figure 3A,B).

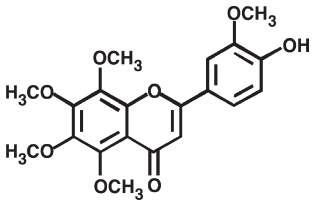

FIGURE 1: Chemical structure of 4′-demethylnobiletin.

4′-Demethylnobiletin Stimulates CRE-Mediated Transcription by Activating the PKA/MEK/ERK-Dependent Signaling in Cultured Hippocampal Neurons. The activation of PKA and ERK induces nuclear translocation and subsequent activation of RSK2, which in turn phosphorylates CREB to stimulate CRE-dependent transcription (*11*). Nobiletin enhances CRE-mediated transcription in PC12D cells (*25*, *26*) and hippocampal neurons (unpublished observations). To test the possibility that the 4′-demethylated metabolite could stimulate CRE-mediated transcription, we employed a reporter gene assay. We observed that this compound appreciably enhanced CRE-mediated transcription at concentrations of 5−50 μM in cultured hippocampal neurons [$F_{(4,25)} = 7.6127$, $p = 0.0004$ by one-way ANOVA] (Figure 4A), indicating the concentration dependency of this compound on CRE-mediated transcription in the hippocampal neurons. Furthermore, consistent with the results from Western blot experiments, 4′-demethylnobiletin-induced CRE-mediated transcription was abolished by both 10 μM H-89 and 5 μM U0126, demonstrating further the involvement of the PKA/MEK/ERK-dependent pathway in the stimulation of CRE-mediated transcription in the hippocampal neurons [$F_{(3,20)} = 31.658$, $p < 0.0001$ by one-way ANOVA] (Figure 4B).

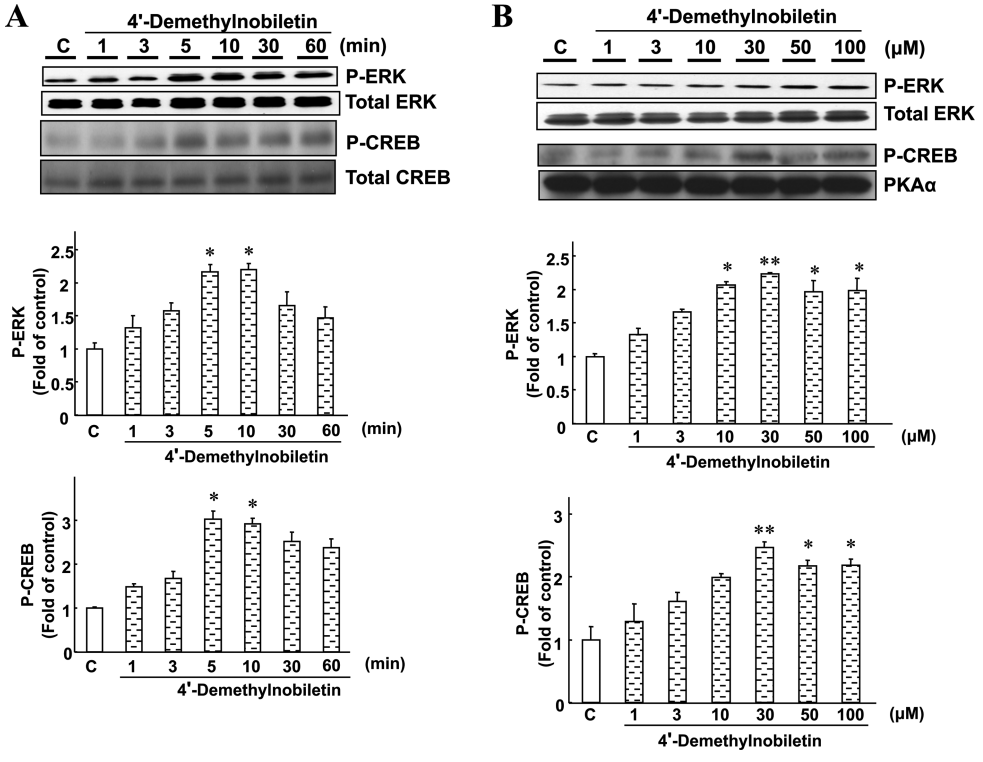

FIGURE 2: (A) Time- and (B) concentration-dependent effects of 4′-demethylnobiletin on the phosphorylation of ERK and CREB in cultured rat hippocampal neurons. Hippocampal neuronal cells plated at a density of 1×10^6 cells/35 mm dish were cultured for 10−14 days. Cells were then stimulated with 4′-demethylnobiletin at 30 μM for the indicated times and for 10 min at the concentrations specified in the figure. Western blot analyses were performed using anti-phospho-ERK and anti-phospho-CREB antibodies. Blots were then stripped and reprobed with anti-ERK, anti-CREB, and anti-PKAα antibodies to confirm that an equal amount of protein was loaded in each lane. The bottom panels correspond to the densitometric analysis of the phosphorylated ERK and CREB normalized to the loading controls of panels A and B, respectively. Values are means ± SEM ($n = 3$). $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisks) by post hoc.

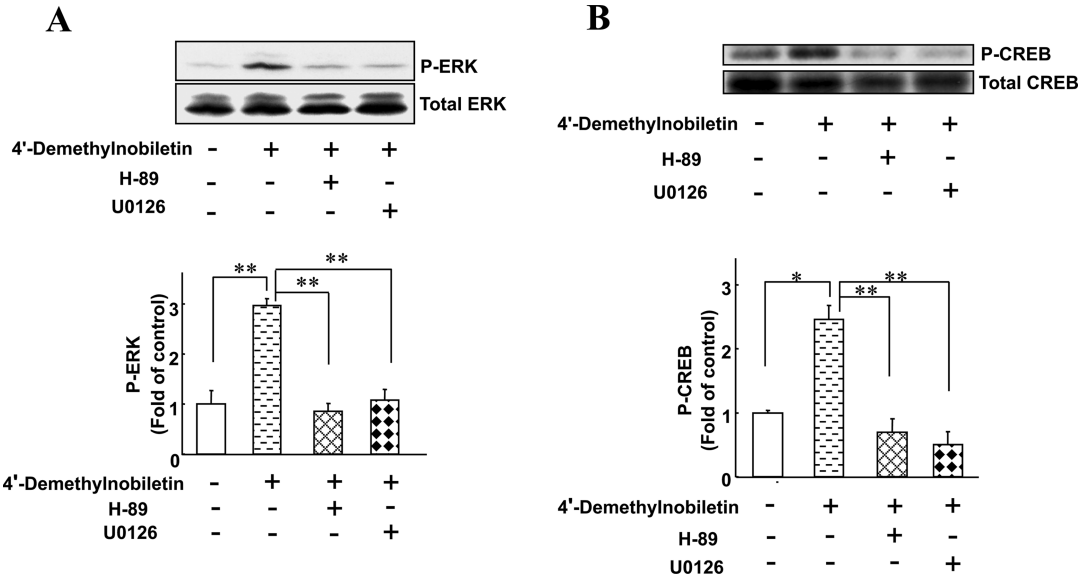

FIGURE 3: 4′-Demethylnobiletin activates a PKA/MEK/ERK signaling pathway in cultured rat hippocampal neurons. Hippocampal cultures were treated with vehicle, 30 μM 4′-demethylnobiletin for 5 min alone, and paired with a 30 min pretreatment with 10 μM H-89 (a PKA inhibitor) and 10 μM U0126 (a MEK inhibitor), respectively. Western blot analyses were performed using anti-phospho-ERK (A) and anti-phospho-CREB (B) antibodies, followed by reprobing with anti-ERK and anti-CREB antibodies, respectively. The bottom panels correspond to the densitometric analysis of the phosphorylated ERK and CREB normalized to the total ERK and CREB of panels A and B, respectively. Values are means ± SEM ($n = 3$). $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisks) by post hoc.

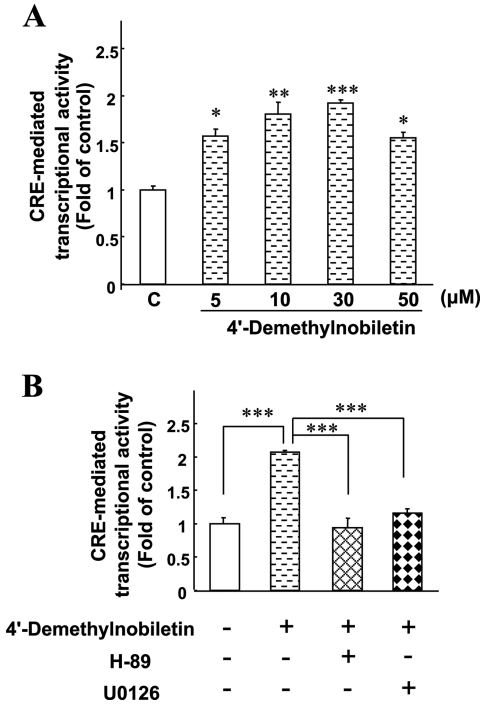

FIGURE 4: Effects of 4′-demethylnobiletin on CRE-mediated transcription in cultured rat hippocampal neurons. Hippocampal neurons plated at a density of 8×10^4 cells/48-well plate were transfected with a luciferase reporter construct showing CRE-mediated transcription for 16 h. Following transfection, neurons were treated with different concentrations of 4′-demethylnobiletin for 8 h (A) or treated with vehicle, 30 μM 4′-demethylnobiletin alone, for 8 h and paired with a 30 min pretreatment with 10 μM H-89 and 5 μM U0126 (B), respectively. Data are means ± SEM ($n = 6$). $P < 0.05$ (one asterisk), $P < 0.01$ (two asterisks), and $P < 0.001$ (three asterisks) by post hoc.

4′-Demethylnobiletin Reverses MK-801-Induced Learning Impairment in Mice. To ascertain the in vivo beneficial effects of 4′-demethylnobiletin on learning impairment, we studied the contextual fear conditioning paradigm in mice, as this protocol elicits robust associative learning (*6*, *16*, *27*). Also, fear conditioning is blocked by the NMDA receptor antagonist MK-801 (*16*). We therefore examined the effects of repeated treatment with 4′-demethylnobiletin (10 or 50 mg/kg, ip) for 7 days on MK-801-induced impairment of contextual fear learning. Treatment with MK-801 or 4′-demethylnobiletin did not significantly alter the freezing behavior during training [treatment × shock event interaction $F_{(6.96)} = 2.094$, $p = 0.0609$] (Figure 5A), while mice treated with MK-801 exhibited less freezing than control mice in the test session performed 24 h after training. Interestingly, repeated administration with 4′-demethylnobiletin (10 or 50 mg/kg) dose-dependently reversed the MK-801-induced learning deficits [$F_{(3,48)} = 12.450$, $p < 0.0001$ by one-way ANOVA] (Figure 5B).

To examine whether 4′-demethylnobiletin specifically affects freezing behavior, we measured the horizontal activities of mice treated with 4′-demethylnobiletin (10 or 50 mg/kg, ip) for 7 days in the open-field test. Repeated treatment with 4′-demethylnobiletin did not affect the horizontal activities of mice [$F_{(2,18)} = 0.0355$, $p = 0.9652$ by one-way ANOVA] (Figure 5C), suggesting that 4′-demethylnobiletin reversed the MK-801-induced learning deficits without affecting the mobility of mice.

4′-Demethylnobiletin Reverses MK-801-Induced Inhibition of Learning-Elicited Activation of ERK in the Hippocampus of Mice. An increase in the level of ERK activation during fear conditioning is a unique consequence of associative learning (*6*). It has been reported that the level of hippocampal ERK2 (p42 MAPK) activation is significantly increased 1 h after training of the contextual fear conditioning and that treatment with MK-801 or AP5 blocks both the associative learning and the activation of ERK in the hippocampus (*6*, *14*). Thus, we examined the effects of 4′-demethylnobiletin on the MK-801-induced inhibition of ERK activation after training of the contextual fear conditioning. The level of phosphorylation of ERK2 was increased in the hippocampus 1 h after fear conditioning;

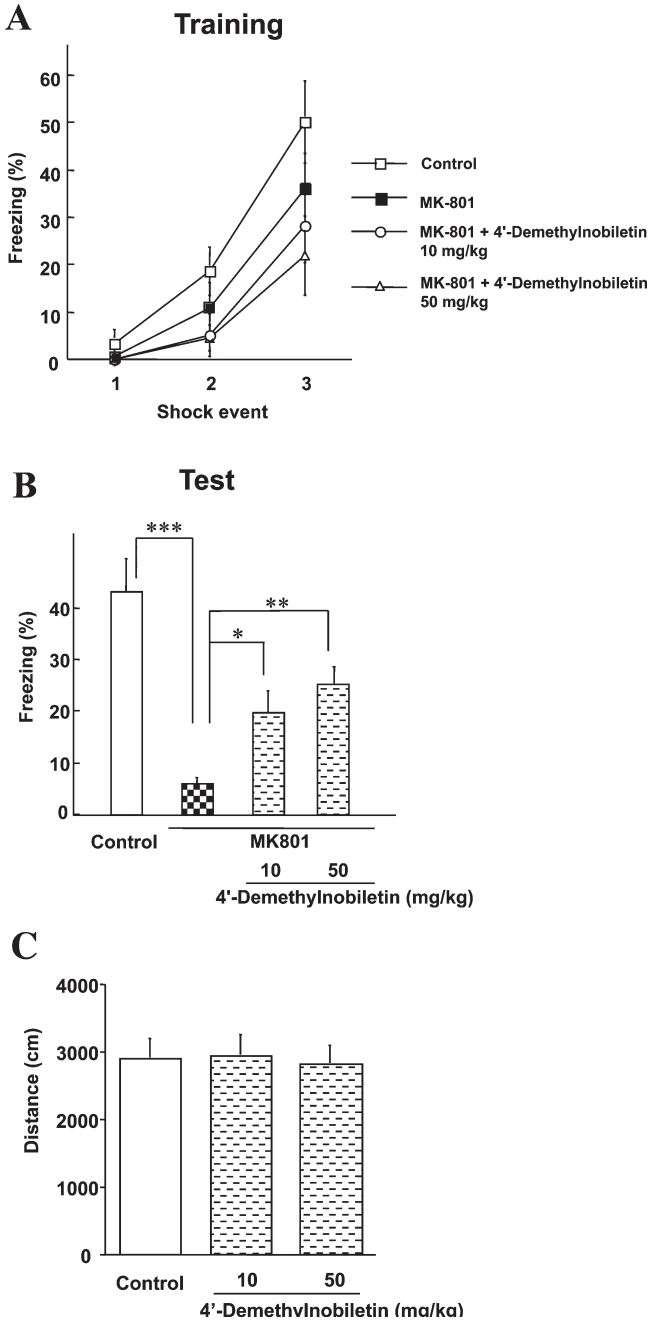

FIGURE 5: 4′-Demethylnobiletin reverses MK-801-induced learning impairment in the contextual fear conditioning paradigm. 4′-Demethylnobiletin (10 or 50 mg/kg, ip) was injected once daily for seven consecutive days (i.e., days 1−7). On day 7, 4′-demethylnobiletin (10 or 50 mg/kg, ip) and MK-801 (0.08 mg/kg, sc) were administered 40 and 30 min before training, respectively. (B) The test session was performed 24 h after training. Values are means ± SEM (Control, $n = 13$; MK-801, $n = 13$; MK-801 + 4′-Demethylnobiletin 10 mg/kg, $n = 13$; MK-801 + 4′-Demethylnobiletin 50 mg/kg, $n = 13$). $P < 0.05$ (one asterisk), $P < 0.01$ (two asterisks), and $P < 0.001$ (three asterisks) by post hoc. (C) Effects of repeated treatment with 4′-demethylnobiletin on horizontal activities in the open-field test. 4′-Demethylnobiletin (10 or 50 mg/kg, ip) was injected once daily for seven consecutive days (i.e., days 1−7). Horizontal activities were measured 24 h after the last injection. Values are means ± SEM (Control, $n = 7$; 4′-Demethylnobiletin 10 mg/kg, $n = 7$; 4′-Demethylnobiletin 50 mg/kg, $n = 7$).

however, no detectable change in ERK1 (p44 MAPK) phosphorylation was observed (Figure 6A,B). Treatment with MK-801 for 30 min prior to the training blocked the learning-induced ERK2

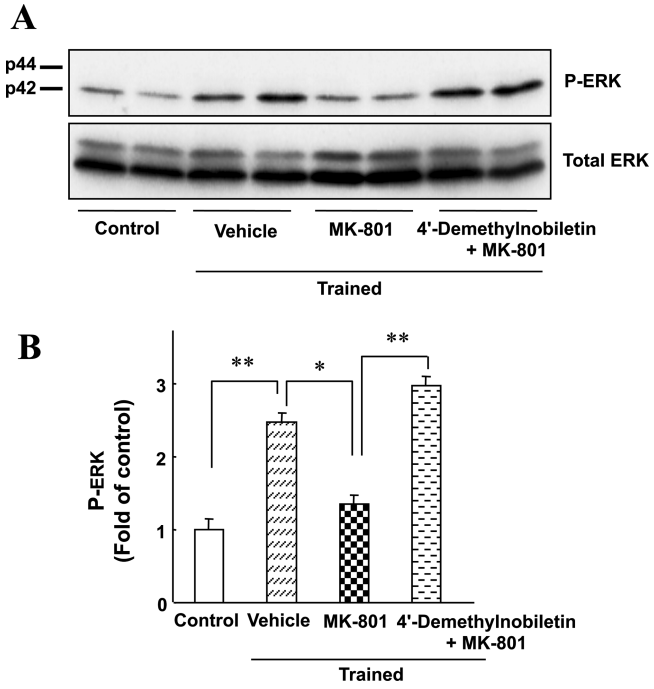

FIGURE 6: 4′-Demethylnobiletin reverses MK-801-induced inhibition of learning-associated activation of ERK in the hippocampus. (A) Representative data on the phosphorylated ERK level for mice sacrificed 1 h after being trained in the contextual fear conditioning task. Following probing with the antibody specific to phosphorylated ERK, blots were reprobed with anti-ERK antibody. (B) Densitometric analysis of the changes in ERK phosphorylation for mice sacrificed 1 h after being trained. Values are means ± SEM (Control, $n = 5$; Vehicle (trained), $n = 5$; MK-801 (trained), $n = 5$; MK-801 + 4′-Demethylnobiletin (trained), $n = 6$). $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisks) by post hoc.

activation. These results suggest the involvement of NMDA receptor activation in the process of enhanced ERK2 phosphorylation observed 1 h after contextual fear conditioning. Strikingly, repeated treatment with 4′-demethylnobiletin (50 mg/kg, ip) for 7 days completely reversed the MK-801-induced inhibition of ERK activation in the hippocampus normally observed 1 h following the contextual fear conditioning [$F_{(3,17)} = 11.817$, $p = 0.0002$ by one-way ANOVA] (Figure 6A,B).

4′-Demethylnobiletin Reverses MK-801-Induced Inhibition of NMDA-Stimulated Phosphorylation of ERK and PKA Substrates in Cultured Hippocampal Neurons. We further examined whether 4′-demethylnobiletin could rescue the MK-801-induced inhibition of NMDA-stimulated phosphorylation of ERK in cultured hippocampal neurons. Treatment with 10 μM NMDA for 15 min appreciably elevated the levels of phosphorylated ERK in rat hippocampal neurons in culture, whereas pretreatment with 10 μM MK-801 blocked the enhanced phosphorylation of ERK. Notably, the inhibitory effect was prevented when 4′-demethylnobiletin was added prior to treatment with MK-801, to rescue the erstwhile elevated level of the phosphorylated proteins (Figure 7). In addition, treatment with 30 μM 4′-demethylnobiletin also rescued MK-801-induced inhibition of NMDA-stimulated phosphorylation of PKA substrates in cultured hippocampal neurons (Figure 7).

4′-Demethylnobiletin Is Detected in the Brains of Mice after Acute Intraperitoneal Administration. To obtain the direct evidence that 4′-demethylnobiletin could reach the brain to rescue the MK-801-induced memory impairment, 4′-demethylnobiletin (50 mg/kg) was ip administered to mice followed by

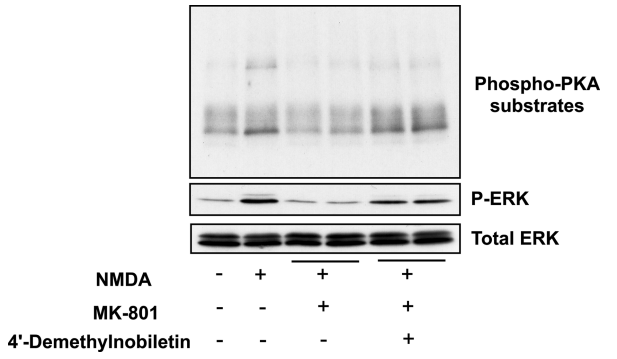

FIGURE 7: 4′-Demethylnobiletin reverses MK-801-induced inhibition of NMDA-stimulated phosphorylation of ERK and PKA substrates in cultured rat hippocampal neurons. Hippocampal neuronal cells plated at a density of 1×10^6 cells/35 mm dish were cultured for 10−14 days. Cultures were then treated with vehicle, 10 μM NMDA for 15 min, 10 μM NMDA for 15 min preceded by an only 30 min treatment with 10 μM MK-801, and a 30 min pretreatment with 30 μM 4′-demethylnobiletin before the addition of MK-801. Western blot analyses were performed using anti-phospho-(Ser/Thr) PKA substrate and anti-phospho-ERK antibodies, followed by reprobing with anti-ERK antibody. Similar results were obtained from two independent experiments.

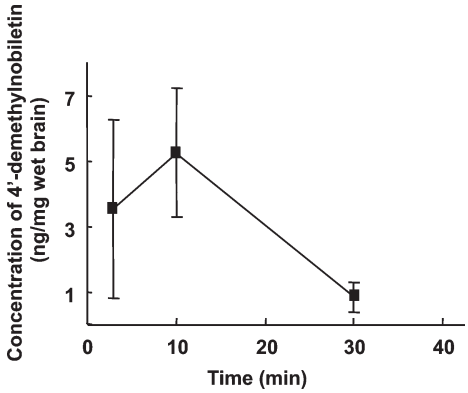

FIGURE 8: Time course of 4′-demethylnobiletin bioavailability in the brain after acute ip injection into the mice. Mice were treated once with 4′-demethylnobiletin (50 mg/kg, ip). Then, mice were sacrificed 3, 10, and 30 min after the injection, followed by immediate collection of the brain. The concentrations of 4′-demethylnobiletin in these brain samples collected at the indicated time points were measured by HPLC/UV. Values are means ± SEM (n = 4).

brain samples being analyzed for the detection of the compound. We successfully detected 4′-demethylnobiletin in the brain by using our HPLC/UV system. 4′-Demethylnobiletin was detected 3 min after its administration. Thereafter, its concentration reached the highest level 10 min after the injection and markedly declined after an additional 20 min (Figure 8), indicating that 4′-demethylnobiletin can exist in the brain and thereby may exert the biological effects observed in our in vitro study.

DISCUSSION

The biological effects of the metabolites of nobiletin in the brain have so far been unaddressed. In this study, we present the first evidence that a major 4′-demethylated metabolite of nobiletin not only activated the PKA/ERK/CREB signaling in cultured hippocampal neurons but also improved the NMDA receptor antagonist-induced learning impairment, accompanied by preventing an inhibition of learning-elicited ERK activation in the hippocampus of mice. It was further shown that 4′-demethylnobiletin reached the brain after acute ip administration. Our results thus suggest that the 4′-demethylated metabolite of nobiletin exhibits both in vitro and in vivo biological actions similar to those of nobiletin.

It is well-known that cAMP activates Rap1, a small GTP-binding protein in the Ras family, which serves as a selective activator of B-Raf, in a PKA-dependent manner, to stimulate B-Raf activity leading to activation of ERK (*8*, *28*, *29*). The activation of ERK is required to induce nuclear translocation and the subsequent activation of RSK2, which in turn phosphorylates CREB to stimulate CRE-dependent transcription (*11*). Our results evidently showed that 4′-demethylnobiletin enhanced not only the phosphorylation of PKA substrates, MEK and ERK, but also the phosphorylation of the downstream target, CREB, indicating a stimulatory effect of this compound on PKA and MEK signaling in the hippocampal neurons. In agreement with the observations, we further demonstrated that the phosphorylation of ERK and CREB induced by 4′-demethylnobiletin was abolished by U0126, a MEK inhibitor, and H-89, a PKA inhibitor, and that both of the inhibitors also completely suppressed 4′-demethylnobiletin-induced CRE-mediated transcription in the hippocampal neurons. In addition, the anti-phospho-PKA substrate antibody used for this study recognizes the phosphorylated substrates by other Arg-directed kinases, including PKC and Akt. Thus, our data cannot completely exclude the possibility that 4′-demethylnobiletin may stimulate protein phosphorylation by other members of Arg-directed kinases. Therefore, these results suggest that like nobiletin (*25*, *26*), its 4′-demethylated derivative also activates PKA/ERK/CREB signaling, thereby stimulating CRE-mediated transcription in the cultured hippocampal neurons.

Because the in vivo ERK activation could play a role in learning and memory formation, we next explored the in vivo effects of 4′-demethylnobiletin in the contextual fear conditioning paradigm. Context-dependent conditioning is used as a variant to assess the hippocampus-dependent form of associative learning (*30*, *31*). In this study, one of the most important findings is that 4′-demethylnobiletin improved the MK-801-induced impairment of the contextual fear conditioning type of associative learning accompanied by rescuing an inhibition of learning-induced ERK activation in the hippocampus of mice, like nobiletin (*19*). How does 4′-demethylnobiletin reverse MK-801-induced learning impairment in mice? We suggest that PKA and PKC may be utilized to couple NMDA receptors, and that each pathway may be capable of activating ERK signaling associated with learning (*31*). Thus, 4′-demethylnobiletin, with its ability to stimulate PKA/ERK/CREB signaling, might reverse MK-801-induced inhibition of learning-associated ERK activation and subsequent learning deficits. Moreover, since nobiletin can inhibit phosphodiesterase (PDE) activity to increase the intracellular cAMP content in cultured cells (*25*), we speculate that 4′-demethylnobiletin-induced PKA activation might have occurred, at least in part, due to inhibition of PDE. In support of this notion, a previous study demonstrates that inhibition of PDE4 improves memory impairment associated with NMDA receptor antagonism (*32*). Consistent with the observations from in vivo experiments, we showed that 4′-demethylnobiletin indeed reversed MK-801-induced inhibition of NMDA-stimulated phosphorylation of ERK and PKA substrates in cultured hippocampal neurons. Taken together, it is reasonable to consider that 4′-demethylnobiletin, with its ability to stimulate CRE-mediated

transcription via cAMP/PKA/ERK signaling as described above, could produce long-lasting effects on learning impairment by modulating transcription of target genes, including those involved in synapse formation, neuronal survival (BDNF and Bcl-2), and long-term memory (C/EBP) (*33*, *34*).

For drugs to be effective in the treatment of neurodegenerative diseases, including AD, bioavailability to the brain is of great importance. Therefore, an inability to cross the blood−brain barrier could limit the therapeutic applications of a drug compound (*35*). Thus, one might argue whether 4′-demethylnobiletin could actually reach the brain to reverse the MK-801-induced learning impairment in mice. In this study, we show that 4′-demethylnobiletin existed in the brains of mice after acute ip administration of the compound. Therefore, it is possible to interpret that, upon ip injection of 4′-demethylnobiletin, this compound crosses the blood−brain barrier to reach the brain, thereby exerting its effect to rescue MK-801-induced learning impairment in mice.

Dysfunction of NMDA receptor-mediated signaling has also been implicated in neurodegenerative disease of the Alzheimer's type. Application of Aβ reduces synaptic NMDA receptors accompanied by a persistent depression of NMDA receptor currents in cultured neurons. Aβ also inhibits NMDA receptor-mediated biochemical signaling, including transcriptional activation through phosphorylation of CREB (*36*, *37*), which is important for learning and memory, although some reports have shown that NMDA receptor activation not only promotes neuronal Aβ production (*38*) but also mediates tau-induced neurotoxicity (*39*) in neuronal culture systems. Furthermore, a very recent study has demonstrated that soluble Aβ dimers isolated directly from Alzheimer's brains induce their effects by perturbing glutamatergic synaptic transmission (*40*). Accordingly, these findings strengthen the idea that dysregulation of NMDA receptor-mediated signaling may initiate AD pathological alterations. Recently, we have also found that 4′-demethylnobiletin reverses Aβ-induced inhibition of glutamate-stimulated PKA/CREB signaling in cultured hippocampal neurons (unpublished observations). Thus, it would be of particular interest to examine whether the beneficial effects of 4′-demethylnobiletin in vitro might be translated into in vivo AD models.

The biological activity of 4′-demethylnobiletin in the brain is quite similar to that of nobiletin as described above. Such a beneficial effect of 4′-demethylnobiletin raises the possibility that the resemblance of their underlying mechanisms could be attributable to their structural similarities. Because the knowledge of in vivo biotransformation of nobiletin is indispensable for an improved understanding of its biological effects and bioactive metabolites could prolong the effects of the parent compound, we emphasize the 4′-demethylated derivative of nobiletin as a main in vivo metabolite. In this study, we demonstrate that like nobiletin, its major 4′-demethylated metabolite also has the ability to stimulate PKA/ERK/CREB-dependent signaling in hippocampal neurons and reverses learning impairment associated with NMDA receptor antagonism in mice. Therefore, this study raises two possibilities: (1) 4′-demethylnobiletin can cross the blood−brain barrier to reach the brain and thereby itself regulate memory functions, and (2) 4′-demethylnobiletin, after being metabolized from nobiletin, can act alongside to prolong the in vivo biological activity even after nobiletin is substantially biotransformed.

In conclusion, in this study, we show that a major 4′-demethylated in vivo metabolite of nobiletin stimulates the phosphorylation of ERK and CREB and CRE-mediated transcription by activating a PKA/MEK/ERK pathway in cultured hippocampal neurons. More importantly, this compound exists in the brain to reverse memory impairment associated with NMDA receptor antagonism via stimulation of ERK signaling in mice. Considering the potential of 4′-demethylnobiletin to act in both in vitro and in vivo systems, it is quite plausible that this compound might become a useful agent in improving cognitive dysfunction associated with not only NMDA receptor−ERK signaling impairment but also other dysregulated pathways involving NMDA receptors and cAMP/PKA/CREB signaling. Therefore, it could be worthwhile to consider the citrus polymethoxyflavone compound, including nobiletin, and its metabolite, 4′-demethylnobiletin, as potential candidate drugs for the treatment of cognitive dysfunction associated with NMDA receptor−ERK signaling impairment.

SUPPORTING INFORMATION AVAILABLE

Effects of 4′-demethylnobiletin on phosphorylation of PKA substrates in cultured rat hippocampal neurons (Figure 1), illustration of the synthetic scheme of 4′-demethylnobiletin (Figure 2), and procedures for the synthesis of 4′-demethylnobiletin. This material is available free of charge via the Internet at http://pubs.acs.org.

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