JPET Fast Forward. Published on February 8, 2007 as DOI: 10.1124/jpet.106.117010

Nobiletin, a citrus flavonoid, reverses learning impairment associated with NMDA receptor antagonism by activation of ERK signaling

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Running title: Effects of nobiletin on MK-801-induced learning impairment

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Text page: 35

Tables: 0

Figures: 5

References: 37

Words: Abstract: 198/250

Introduction: 349/750

Discussion: 1093/1500

Recommended section: Neuropharmacology

Abbreviations: ERK, extracellular signal-regulated kinase; PKA, protein kinase A; NMDA, *N*-methyl-D-aspartate; PDE, phosphodiesterase; CRE, cAMP response element; CREB, cAMP response element binding protein; MEK, mitogen-activated protein kinase kinase

Abstract

Recent studies have indicated that learning-induced activation of ERK signaling via NMDA receptors is required for consolidation of the resultant learning. These findings raise an idea that control of ERK signaling may be a potential target for treatment of cognitive dysfunction. Our recent studies have demonstrated that nobiletin, a polymethoxylated flavone from *Citrus depressa*, enhances cAMP/PKA/ERK signaling in cultured rat hippocampal neurons and PC12D cells. Here we, for the first time, present the evidence that this natural compound reverses learning impairment associated with NMDA receptor antagonism by activation of ERK in the hippocampus: Treatment with 50 mg/kg nobiletin reversed the NMDA receptor antagonist MK-801-induced learning impairment in mice. Western blot analysis also showed that nobiletin reversed MK-801-induced inhibition of learning-associated ERK activation in the hippocampus of the animals. Furthermore consistent with these results, in cultured rat hippocampal neurons, nobiletin restored MK-801-induced impairment of NMDA-stimulated phosphorylation of ERK in a concentration-dependent manner. Taken together, the present study suggests that compounds which activate ERK signaling improve cognitive deficits associated with NMDA receptor hypofunction, and that nobiletin may give us a new insight into therapeutic drug development for neurological disorders exhibiting cognitive impairment accompanied by a hypofunction of NMDA receptor-ERK signaling.

Introduction

N-methyl-D-aspartate (NMDA) receptors are widely distributed in the brain; their density is highest in the hippocampal CA1 region (Monyer et al., 1994). It has been shown that NMDA receptors are very important in the regulation of synaptic plasticity and the process of learning and memory (Riedel et al., 2003). Activation of NMDA receptors can initiate second messenger signaling pathways, including cAMP-dependent signaling pathway that plays crucial roles in a positive regulation of neurite outgrowth via extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) activation (Vossler et al., 1997), glutamatergic neurotransmission (Banke et al., 2000), and the hippocampal long-term potentiation associated with learning and memory (Abel et al., 1997). Recent studies indicate that learning-induced activation of ERK signaling via NMDA receptors is required for consolidation of the resultant learning (Atkins et al., 1998; Cammarota et al., 2000; Athos et al., 2002; Levenson et al., 2004; Chwang et al., 2006). Reversely, in vivo blockade of NMDA receptors or inhibition of ERK signaling results in impairment of associative learning (Atkins et al., 1998; Kim et al., 1991). Furthermore, long-lasting impairment of associative learning after repeated administration of phencyclidine, a noncompetitive NMDA receptor antagonist, is correlated with a dysfunction of ERK signaling

(Enomoto et al., 2005). Accordingly, it is plausible that control of ERK signaling is a potential target for treatment of neurological disorders exhibiting cognitive dysfunction.

Large numbers of compounds from natural resources have provided not only useful pharmacological tools (Furukawa et al., 1993; Obara et al., 2002; Ohizumi, 1997) but also novel leading compounds for drug development (Liu, 1993). Flavonoids form a large family of natural products and are widely distributed in the plant kingdom. In the course of our survey of substances having the activity to potentiate cAMP/PKA/ERK signaling to repair dysregulated neuronal functions from natural resources, we successfully found nobiletin, a polymethoxylated flavone from *Citrus depressa* (Fig. 1), as a natural compound that enhances cAMP/PKA/ERK signaling in PC12D cells and cultured hippocampal neurons (Nagase et al., 2005a, b). Here we describe the first evidence that nobiletin reverses the learning impairment associated with NMDA receptor antagonism by activation of ERK signaling.

Materials and Methods

Animals.

Male ddY mice (8 weeks old) were obtained from Nippon SLC (Hamamatsu, Japan). Animals were housed in cages with free access to food and water under the condition of constant temperature (23 ± 1°C) and humidity (55 ± 5%) and adapted to a standard 12-h light/12-h dark cycle (light cycle: 9:00 - 21:00). The procedures used in this study 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 US National Institute of Health.

Drugs.

Nobiletin was extracted and isolated as described previously (Nagase et al., 2005a), and suspended in 0.5 % Tween 80 solution. MK-801 (Sigma, St. Louis, MO) was dissolved in saline.

Step-through passive-avoidance task.

The training trial and retention test of the passive avoidance task were conducted in a plexiglas box which contained dark (25 x 25 x 25 cm) and light (14 x 10 x 25 cm) compartments. The floor rods in the dark compartment were connected to an electronic

stimulator (Nihon Kohden, Tokyo, Japan). The training trial was started by placing the mouse on the light compartment of the box. When the mouse completely entered the dark compartment, an electric shock (0.4 mA, 1 s) was supplied to the floor bars. Twenty-four hr after training, each mouse was placed in the light compartment and step-through latency was recorded until 300 s had elapsed (retention test). MK-801 (0.1 mg/kg, s.c.) was given 30 min before the training trial of the passive avoidance task. To examine the effects of single treatment with nobiletin, nobiletin (25-50 mg/kg, i.p.) or vehicle (0.5 % Tween 80) was injected 10 min prior to MK-801. To examine the effects of repeated treatment with nobiletin, nobiletin (50 mg/kg, i.p.) was injected once daily for 6 or 7 consecutive days (i.e. day 1-6 or day 1-7). On day 7, vehicle or nobiletin (50 mg/kg) was injected 10 min prior to MK-801, which was given 30 min before training of the passive avoidance task. The dose of MK-801 (0.1 mg/kg) was selected on the basis of a previous study, which showed that MK-801 (0.03-0.1 mg/kg) dose-dependently impaired the passive avoidance performance (Maurice et al., 1994). The doses of nobiletin (25-50 mg/kg) and the administration route (i.p.) were chosen on the basis of our previous study showing the protective effect of nobiletin on β-amyloid peptide (Aβ)-induced impairment of learning and memory (Matsuzaki et al., 2006).

Contextual fear conditioning.

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 were repeated for a total of three shocks. After the last shock, animals were allowed to context for an additional 1 min prior to removal 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 hr after fear conditioning and scored for freezing for 5 min. Freezing behavior was measured by observing the animals every 5 s. Nobiletin (50 mg/kg, i.p.) was injected once daily for 7 consecutive days (day 1-7). On day 7, vehicle or nobiletin (50 mg/kg) was injected 10 min prior to saline or MK-801 (0.08 mg/kg, s.c.), which was given 30 min before training of contextual fear conditioning paradigm. The dose used (0.08 mg/kg) was chosen on the basis of a previous study showing that MK-801 (0.08 mg/kg) reduced both contextual associative learning and activation of ERK during fear conditioning (Atkins et al., 1998).

To examine the effects of nobiletin on sensitivities to foot shocks, threshold values were determined by electrical stimulation intensity leading to stereotypic responses as described previously (Barad et al., 1998). Mice were injected with nobiletin (10-50 mg/kg, i.p.) once

daily for 7 consecutive days (i.e. day 1-7). On day 7, vehicle or nobiletin (10-50 mg/kg) was administered 10 min prior to MK-801 (0.1 mg/kg, s.c.), which was given 30 min before the nociception test. Each mouse was placed in a chamber with a floor of metal bars and then exposed to 2 s shocks every 30 s in order of increasing intensity (shocks started at 0.05 mA and were increased by 0.05 after each interval). Mice were scored for their first visible response to the shock (flinch), their first extreme motor response (run/jump), and their first vocalized distress (vocalize).

Western blot analysis.

Western blot analysis was performed as described previously (Thiels et al., 2002) with a minor modification. Mice were treated with nobiletin (50 mg/kg) once daily for 7 consecutive days (i.e. day 1-7). On day 7, nobiletin was administered 10 min prior to MK-801 (0.1 mg/kg, s.c.), which was given 30 min before the training trial of the passive avoidance task. Immediately and 30 min after training, mice were killed by decapitation, and the brains were immediately removed. To examine the effects of nobiletin on the basal phospho-ERK levels, mice were killed before training. Hippocampi were homogenized in ice-cold homogenation buffer (10 mM HEPES-OH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM *p*-AMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml

antipain, 2 μg/ml chymostain). The homogenate was centrifuged at 2000 x *g* at 4˚C for 10 min. The obtained nuclear pellet was resuspended in 40 μl of ice-cold extraction buffer (10 mM HEPES, pH 7.0, 450 mM NaCl, 5 mM EDTA, 0.05% SDS, 1% NP-40, 1 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM p-AMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml antipain, 2 μg/ml chymostain), and incubated on ice for 15 min. The mixture was centrifuged at 12,000 x g for 10 min, and the supernatant was collected. Equal amounts of protein (20 μg) were subjected to SDS-polyacrylamide gel electrophoresis (12.5% gels), and the blotted membrane was blocked in TBST buffer containing 5% skim milk for 1 hr at room temperature. The membrane was then incubated with anti-phospho-ERK (Thr 202/Tyr 204) antibody (Cell Signaling Technology, Beverly, MA) in 2% BSA/TBST buffer overnight at 4˚C and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cell Signaling Technology) for 2 hr at room temperature. Following stripping of the antibody, the membrane was reprobed with anti-ERK antibody (Promega, Madison, WI). Immunoreactivities were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce). The band intensities were quantitatively analyzed using SCION image software. To evaluate the ERK activation, the phospho-ERK levels were normalized to the total ERK levels in the same membranes.

Preparation of neuronal cultures.

Hippocampal neuron culture was carried out as described previously (Matsuzaki et al., 2006). In brief, under ether anesthesia of an 18-day pregnant Sprague Dawley rat (Japan SLC), embryos were immediately decapitated. The hippocampal neurons were dissociated according to the standard methods with SUMITOMO Nerve Cell Culture System (SUMITOMO BAKELITE), plated on 35-mm culture dish coated with poly-L-lysine at a density of 1 x 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% penicilline-streptomycin, and cultured at 37°C in the medium described above. At 4 days in vitro (D.I.V.), a half of the medium was replaced by the medium containing a mitotic inhibitor, cytosine arabinofuranoside (Ara-C), to minimize glial proliferation. At 14 D.I.V. cultured neurons were treated with nobiletin (3-30 μM) or vehicle in the Ara-C-cotaining medium without B-27 Supplement and L-glutamine for 1 hr, and thereafter 10 μM MK-801 was added to the medium. Following 30 min, cultured neurons were treated with 10 μM NMDA for 15 min and then subjected to Western blot analysis using anti-phospho-ERK (Thr 202/Tyr 204) and anti-phospho-(Ser/Thr) PKA substrate antibodies (Cell Signaling Technology) as

described previously (Matsuzaki et al., 2006).

Statistical analyses.

The results are expressed as means ± S.E.M. The data for the freezing during training of contextual fear conditioning paradigm were analyzed by two-way repeated measures analysis of variance (ANOVA). Other data were analyzed by one-way ANOVA, followed by the Student-Neumann-Keuls test. A level of $p < 0.05$ was considered statistically significant.

JPET Fast Forward. Published on February 8, 2007 as DOI: 10.1124/jpet.106.117010
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Results

Nobiletin reverses MK-801-induced learning impairment.

We used the passive avoidance task, in which animals learn to associate a location with an aversive stimulus (Stubley-Weatherly et al., 1996; Bernabeu et al., 1997; Taubenfeld et al., 2001). We first investigated the effects of a single treatment with nobiletin (25-50 mg/kg, i.p.) on NMDA receptor antagonist MK-801-induced impairment of learning and memory in the passive avoidance test. The treatment with MK-801 (0.1 mg/kg, s.c.) resulted in a decrease in the step-through latency in the retention test performed 24 hr after the training trial (Fig. 2A). Preadministration of nobiletin (50 mg/kg, i.p.) led to a moderate but significant increase in the step-through latency ($F_{(2,47)}$=4.4136, p = 0.0175) (Fig. 2A).

Next, we examined the effects of repeated treatment with nobiletin on MK-801-induced impairment of learning and memory. The training trial of the passive avoidance test was performed on day 7, and the retention test was performed on day 8. Repeated treatment with nobiletin (50 mg/kg, i.p.) for 6 days (i.e. day 1-6) or 7 days (i.e. day 1-7) reversed the MK-801-induced learning and memory deficits by 50% or 90%, respectively ($F_{(2,26)}$=11.292, p = 0.0003) (Fig. 2B).

To ascertain the effects of repeated treatment with nobiletin on MK-801-induced

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impairment of learning and memory, we proceeded to study in another form of associative learning paradigm, contextual fear conditioning (Kim et al., 1991; Logue et al., 1997; Atkins et al., 1998). We first examined the effects of repeated treatment with nobiletin (10-50 mg/kg, i.p., for 7 days) on contextual fear learning in naive mice. Mice injected with either vehicle or nobiletin (10-50 mg/kg) showed similar amounts of freezing behavior during both the training session and test session (treatment x shock event interaction $F_{(2,54)} = 0.314$, $p = 0.8675$; $F_{(2,27)} = 0.6433$, $p = 0.5334$, respectively) (Fig. 3, A and B). Next we examined the effects of repeated treatment with nobiletin (10-50 mg/kg, i.p., for 7 days) on MK-801-induced impairment of contextual fear learning. The treatment with neither MK-801 nor nobiletin affected freezing behavior during training (treatment x shock event interaction $F_{(2,88)} = 0.946$, $p = 0.4664$) (Fig. 3C). In the test session performed 24 hr after training, mice injected with MK-801 showed less freezing behavior than control mice (Fig. 3D). Repeated treatment with nobiletin (10-50 mg/kg) dose-dependently reversed the MK-801-induced learning deficits ($F_{(2,32)} = 5.2196$, $p = 0.0109$) (Fig. 3D).

Because these learning paradigms involved foot shock, which serves as an aversive stimulus, it was important to determine if nobiletin-treated mice differed from vehicle-treated mice in their sensitivities to foot shocks of varying intensities. The stimulus thresholds for

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flinching, running/jumping, and vocalizing for vehicle-treated and nobiletin-treated mice were indistinguishable [stimulus threshold for flinching, running/jumping, vocalizing (mA); control: 0.18 ± 0.02, 0.29 ± 0.03, 0.33 ± 0.05, respectively, n = 7; MK-801: 0.14 ± 0.03, 0.20 ± 0.03, 0.24 ± 0.02, respectively, n = 7; nobiletin 10 mg/kg + MK-801: 0.19 ± 0.01, 0.26 ± 0.03, 0.29 ± 0.03, respectively, n = 7; nobiletin 50 mg/kg + MK-801: 0.19 ± 0.02, 0.28 ± 0.01, 0.31 ± 0.01, respectively, n = 8; flinching: $F_{(2,19)}$=2.5015, p = 0.1086, running/jumping: $F_{(2,19)}$=2.5909, p = 0.1012, vocalizing: $F_{(2,19)}$=2.3249, p = 0.1250]. These results suggest that any differences between vehicle-treated and nobiletin-treated mice in the passive avoidance test and contextual fear conditioning paradigm are not due to altered shock sensitivity.

Nobiletin reverses MK-801-induced inhibition of learning-associated activation of ERK in the hippocampus.

It has been reported that a single passive avoidance training event has been previously shown to activate hippocampal ERK, which is necessary for consolidation of the resultant learning (Cammarota et al., 2000; Alonso et al., 2002). Furthermore, treatment with MK-801 or AP5 blocks both the associative learning and the activation of ERK in the hippocampus (Atkins et al., 1998; Cammarota et al., 2000). Thus, we next examined the effects of nobiletin

on the MK-801-induced inhibition of ERK activation in the hippocampus after the passive avoidance training in mice. The passive avoidance training increased levels of phospho-ERK when measured immediately after training ($F_{(3,14)}$=7.9959, p = 0.0024) (Fig. 4, A and B), whereas the increase in phospho-ERK was not found 30 min after training, indicating that the activation of ERK appears to be rapid and transient following training ($F_{(3,8)}$=1.4373, p = 0.3023) (Fig. 4, C and D). Treatment with MK-801 30 min prior to the passive avoidance training blocked the increase in phospho-ERK which was normally observed immediately after training (Fig. 4, A and B). These results demonstrate that the increase in phospho-ERK observed after the passive avoidance training requires activation of NMDA receptors. Notably repeated administration of nobiletin (50 mg/kg, i.p., for 7 days) completely reversed the MK-801-induced inhibition of ERK activation in the hippocampus normally observed immediately after the passive avoidance training (Fig . 4, A and B). In addition, repeated treatment with nobiletin did not affect the basal phospho-ERK levels (Fig. 4, E and F), suggesting that ERK activation in nobiletin-treated trained mice results from the passive avoidance training.

Nobiletin reverses MK-801-induced inhibition of NMDA-stimulated phosphorylation of

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ERK and PKA substrates in cultured hippocampal neurons.

We further examined the effects of nobiletin on ERK activation in cultured rat hippocampal neurons. Treatment with 10 μM NMDA for 15 min increased the level of phospho-ERK by 900% in cultured rat hippocampal neurons (Fig. 5, A and B). Pretreatment with 10 μM MK-801 completely prevented the 10 μM NMDA-induced increase in phospho-ERK (Fig. 5, A and B). When nobiletin was added prior to treatment with MK-801, the inhibitory effect of MK-801 was completely blocked by 10 or 30 μM of the natural compound, while it showed no effect on the inhibitory action of MK-801 at the concentration of 3 μM, suggesting the concentration-dependency ($F_{(3,12)}$=11.802, p = 0.0007) (Fig. 5, A and B). Furthermore, treatment with nobiletin (3-30 μM) concentration-dependently reversed MK-801-induced inhibition of NMDA-stimulated phosphorylation of PKA substrates in cultured rat hippocampal neurons (Fig. 5C).

Discussion

Learning-associated signaling begins at the plasma membrane, where activation of NMDA receptors leads to influx of Ca^{2+} and engagement of a variety of signaling pathways that ultimately converge on the ERK signaling cascade (Adams and Sweatt, 2002). Upon activation, ERK performs several functions relevant for establishing short and long term memory (Sweatt, 2004). Especially, ERK activation leads to a number of cellular changes associated with the development of long term memory, such as alterations in gene expression and protein synthesis, dendritic spine stabilization, the modulation of ion channels, and changes in receptor trafficking. In the present study, we demonstrated that treatment with nobiletin improved the NMDA receptor antagonist-induced learning impairment, accompanied by preventing an inhibition of learning-associated ERK activation in the hippocampus of mice. It was also shown that nobiletin reversed MK-801-induced impairment of NMDA-stimulated phosphorylation of ERK in cultured rat hippocampal neurons. Given that the activation of ERK in the hippocampus is necessary for consolidation of the resultant learning (Atkins et al., 1998; Cammarota et al., 2000; Athos et al., 2002; Levenson et al., 2004; Chwang et al., 2006), our results suggest that nobiletin reverses MK-801-induced learning deficits by activation of ERK in the hippocampus.

How could nobiletin reverse MK-801-induced inhibition of ERK activation in the hippocampus? We have recently reported that nobiletin inhibits the phosphodiesterase (PDE) activity catalyzing the hydrolysis of cAMP, and thereby increases intracellular cAMP concentration to activate PKA in PC12D cells (Nagase et al., 2005b). Furthermore, nobiletin induces a sustained increase in phosphorylation of MEK and ERK in cultured hippocampal neurons as well as in PC12D cells (Nagase et al., 2005b). 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 (Yao et al., 1998; York et al., 1998; Vossler et al., 1997). Also this compound stimulates CREB phosphorylation and CRE-mediated transcription in a MEK/ERK-dependent signaling cascade (Nagase et al., 2005a, b). Collectively, it is plausible that nobiletin inhibits PDE activity at least at the intracellular local region to activate cAMP/PKA/MEK/ERK signaling cascade, and thereby reverses MK-801-induced inhibition of learning-associated ERK activation and subsequent learning deficits. In support of this interpretation, it was observed that, in cultured rat hippocampal neurons, nobiletin reversed MK-801-induced impairment of NMDA-stimulated phosphorylation of PKA substrates and ERK (Fig. 5).

Because nobiletin itself activates ERK signaling, one might argue that the effects of nobiletin in the present study might be independent of NMDA receptor signaling. Recently, we have found that treatment with 10 μM nobiletin increased the level of phospho-ERK by 300 % at 90 min after treatment in cultured rat hippocampal neurons (Matsuzaki et al., unpublished observation). However, it should be mentioned that, in the present study, treatment with 10 μM NMDA increased the level of phospho-ERK by 900%, and that 10 μM nobiletin completely reversed the inhibitory effect of MK-801, suggesting that nobiletin-induced ERK activation, at least in part, depends on NMDA receptor stimulation. The present findings are consistent with those of the previous study showing a functional interaction between PDE and NMDA receptors in the regulation of learning and memory (Zhang et al., 2000).

In the present study, in the passive avoidance test, both single and repeated administration of nobiletin improved the learning impairment induced by MK-801. It is important to mention here that mice treated with nobiletin for 6 days (i.e. day 1-6) did not receive nobiletin on the training day (i.e. day 7) of the passive avoidance task. Nevertheless, these mice showed increased step-through latency in the retention test performed 24 hr after the training trial. This result indicates that nobiletin has not only acute effects but also chronic

effects against MK-801-induced learning impairment. Considering the ability of nobiletin to trigger an increase in CREB phosphorylation and CRE-dependent transcription via activation of a cAMP/PKA/ERK signaling pathway in cultured rat hippocampal neurons (Matsuzaki et al., unpublished observation), it is possible that the chronic effects of nobiletin may result from alterations in transcription of target genes, including those involved in synapse formation, neuronal survival (BDNF, Bcl-2) and long-term memory (C/EBP) (Shieh et al., 1998; Tao et al., 1998; Taubenfeld et al., 2001).

Cognitive impairment associated with NMDA receptor hypofunction is hypothesized in several neurological disorders including Alzheimer's disease (AD) and schizophrenia. Recent study indicates that application of Aβ promotes endocytosis of NMDA receptors in cultured neurons accompanied by a persistent depression of NMDA-evoked currents and reduced signaling to CREB (Snyder et al., 2005). These findings suggest that prolonged depression of NMDA receptor-mediated transmission may initiate the pathological changes observed in AD. We have recently reported that nobiletin reverses sublethal concentration of $A\beta_{1-42}$-induced reduction in the activity of PKA/CREB-dependent signaling pathway in cultured rat hippocampal neurons (Matsuzaki et al., 2006). This compound also ameliorates Aβ-induced impairment of memory in AD model rats (Matsuzaki et al., 2006). Furthermore,

our preliminary results showed that nobiletin improved learning and memory deficits in human amyloid precursor protein (APP) transgenic mice that overexpress human APP695 harboring the double Swedish/London mutation (Onozuka et al., unpublished observation). Taken together, nobiletin may have the potential to become a novel lead compound for drug development for AD with novel mechanisms of the action.

In addition to its role in learning and memory, the glutamate system has been implicated in schizophrenia. NMDA receptor hypofunction may be critically involved in the etiology or symptoms associated with this disease. Thus, NMDA receptor antagonists such as phencyclidine, MK-801, and ketamine induce psychotic states in normal human volunteers and exacerbate symptoms in patients with schizophrenia (Olney et al., 1999; Javitt and Zukin, 1991). Based on the results of the present study and the previously reported finding that phencyclidine-treated animal, a useful model of schizophrenia, induces a long-lasting impairment of associative learning accompanied by a decrease in learning-associated NMDA-ERK signaling (Enomoto et al., 2005), nobiletin might improve the cognitive impairment associated with schizophrenia through activation of ERK signaling. Furthermore, it is necessary to investigate the beneficial effects of nobiletin on schizophrenia-like positive and negative symptoms in animal models of schizophrenia in the future study.

In conclusion, the present results demonstrate that nobiletin reverses the learning impairment associated with NMDA receptor antagonism by activation of ERK signaling. These data strengthen the idea that activation of ERK signaling in the hippocampus may improve cognitive dysfunction in neurological disorders associated with NMDA receptor hypofunction. The beneficial effects of nobiletin may thus give a new insight into therapeutic drug development for neurological disorders exhibiting cognitive impairment caused by a dysfunction of NMDA receptor-ERK signaling.

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Footnotes

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and the grant from Takeda Science Foundation.

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Legends for Figures

Fig. 1. Chemical structure of nobiletin.

Fig. 2. Nobiletin reverses MK-801-induced learning impairment in the passive avoidance test. (A) Effects of a single treatment with nobiletin on MK-801-induced learning impairment in the passive avoidance test. Nobiletin (25 or 50 mg/kg, i.p.) and MK-801 (0.1 mg/kg, s.c.) were administered 40 and 30 min before the training trial, respectively. The retention test was performed 24 hr after the training trial. Values are means ± S.E.M. (Control, n = 9; MK-801, n = 17; MK-801 + Nobiletin 25 mg/kg, n = 13; MK-801 + Nobiletin 50 mg/kg, n = 20). $^{**}p < 0.01$ vs Control. $^{\#}p < 0.05$ vs MK-801. (B) Effects of repeated treatment with nobiletin on MK-801-induced learning impairment in the passive avoidance test. Nobiletin (50 mg/kg, i.p.) was injected once daily for 6 or 7 consecutive days (i.e. day 1-6 or day 1-7). On day 7, nobiletin (50 mg/kg, i.p.) and MK-801 (0.1 mg/kg, s.c.) were administered 40 and 30 min before the training trial, respectively. The retention test was performed 24 hr after the training trial. Values are means ± S.E.M. (Control, n = 8; MK-801, n = 8; MK-801 + Nobiletin 50 mg/kg (day 1-6), n = 9; MK-801 + Nobiletin 50 mg/kg (day 1-7), n = 12). $^{**}p < 0.01$ vs

Control. #$p < 0.05$, ##$p < 0.01$ vs MK-801.

Fig. 3. Nobiletin reverses MK-801-induced learning impairment in the contextual fear conditioning paradigm. (A and B) Effects of repeated treatment with nobiletin (10-50 mg/kg, i.p., for 7 days) on contextual fear conditioning in naive mice. Nobiletin (10-50 mg/kg, i.p.) was injected once daily for 7 consecutive days (i.e. day 1-7). On day 7, nobiletin (10-50 mg/kg, i.p.) was administered 40 min before training. The test session was performed 24 hr after training. Values are means ± S.E.M. (Control, n = 10; Nobiletin 10 mg/kg, n = 9; Nobiletin 50 mg/kg, n = 11). (C and D) Effects of repeated treatment with nobiletin (10-50 mg/kg, i.p., for 7 days) on MK-801-induced learning impairment in the contextual fear conditioning paradigm. Nobiletin (10-50 mg/kg, i.p.) was injected once daily for 7 consecutive days (i.e. day 1-7). On day 7, nobiletin (10-50 mg/kg, i.p.) and MK-801 (0.08 mg/kg, s.c.) were administered 40 and 30 min before training, respectively. The test session was performed 24 hr after training. Values are means ± S.E.M. (Control, n = 13; MK-801, n = 9; MK-801 + Nobiletin 10 mg/kg, n = 13; MK-801 + Nobiletin 50 mg/kg, n = 13). *$p < 0.05$, **$p < 0.01$ vs Control. #$p < 0.05$, ##$p < 0.01$ vs MK-801.

Fig. 4. Nobiletin reverses MK-801-induced inhibition of learning-associated activation of ERK in the hippocampus. (A) Representative data on phosphorylated ERK level for mice sacrificed immediately after training of the passive avoidance task. Following probing with the antibody specific to phosphorylated ERK, blots were reprobed with anti-ERK antibody. (B) Densitmetric analysis of the changes in ERK phosphorylation for mice sacrificed immediately after training. Values are means ± S.E.M. (Control, n = 4; Vehicle (trained), n = 4; MK-801 (trained), n = 4; MK-801 + Nobiletin (trained), n = 6). *$p < 0.05$ vs Control. #$p < 0.05$ vs Vehicle-treated trained group. $$$p < 0.01$ vs MK-801-treated trained group. (C) Representative data on phosphorylated ERK level for mice sacrificed 30 min after training of the passive avoidance task. (D) Densitmetric analysis of the changes in ERK phosphorylation for mice sacrificed 30 min after training. Values are means ± S.E.M. (Control, n = 3; Vehicle (trained), n = 3; MK-801 (trained), n = 3; MK-801 + Nobiletin (trained), n = 3). (E) Representative data on basal phosphorylated ERK level for mice sacrificed before training of the passive avoidance task. (F) Densitmetric analysis of the changes in ERK phosphorylation for mice sacrificed before training. Values are means ± S.E.M. (Control, n = 4; Nobiletin, n = 3).

Fig. 5. Nobiletin reverses MK-801-induced inhibition of NMDA-stimulated phosphorylation of ERK and PKA substrates in cultured rat hippocampal neurons. (A) The levels of phosphorylated ERK in hippocampal neurons were examined by Western blotting with the antibody specific to phosphorylated ERK. Blots were then stripped and reprobed with anti-ERK antibody. (B) Densitmetric analysis of the levels of phosphorylated ERK in cultured neurons treated with NMDA or NMDA plus MK-801 in the absence and presence of nobiletin. Values are means ± S.E.M. (n = 4). ***p* < 0.01 vs Control. ##*p* < 0.01 vs NMDA-treated group. $$*p* < 0.01 vs NMDA plus MK-801-treated group. (C) The levels of phosphorylated PKA substrates in hippocampal neurons were examined by Western blotting using anti-phospho-PKA substrates antibody. Blots were then stripped and reprobed with anti-PKAα antibody to verify that equal amount of protein was present in each lane. Similar results were obtained from three independent experiments.

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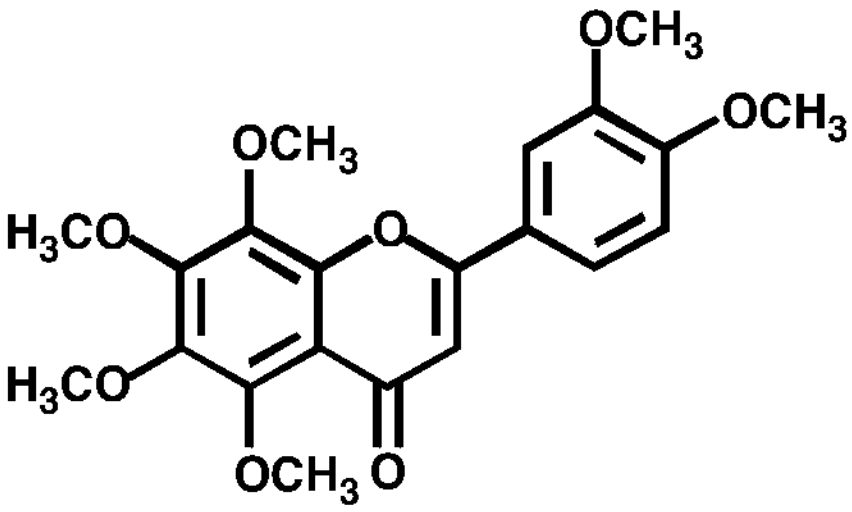

Fig. 1

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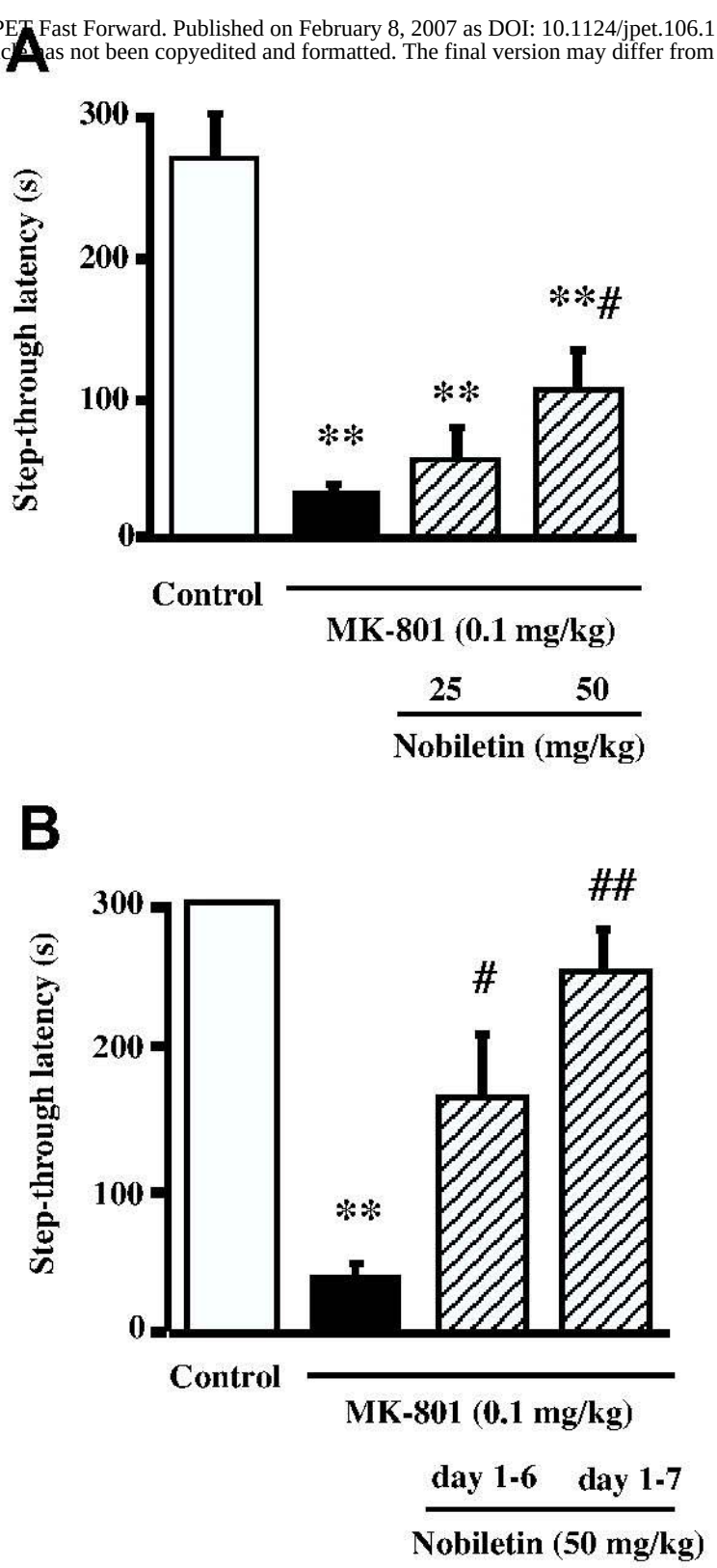

Fig. 2

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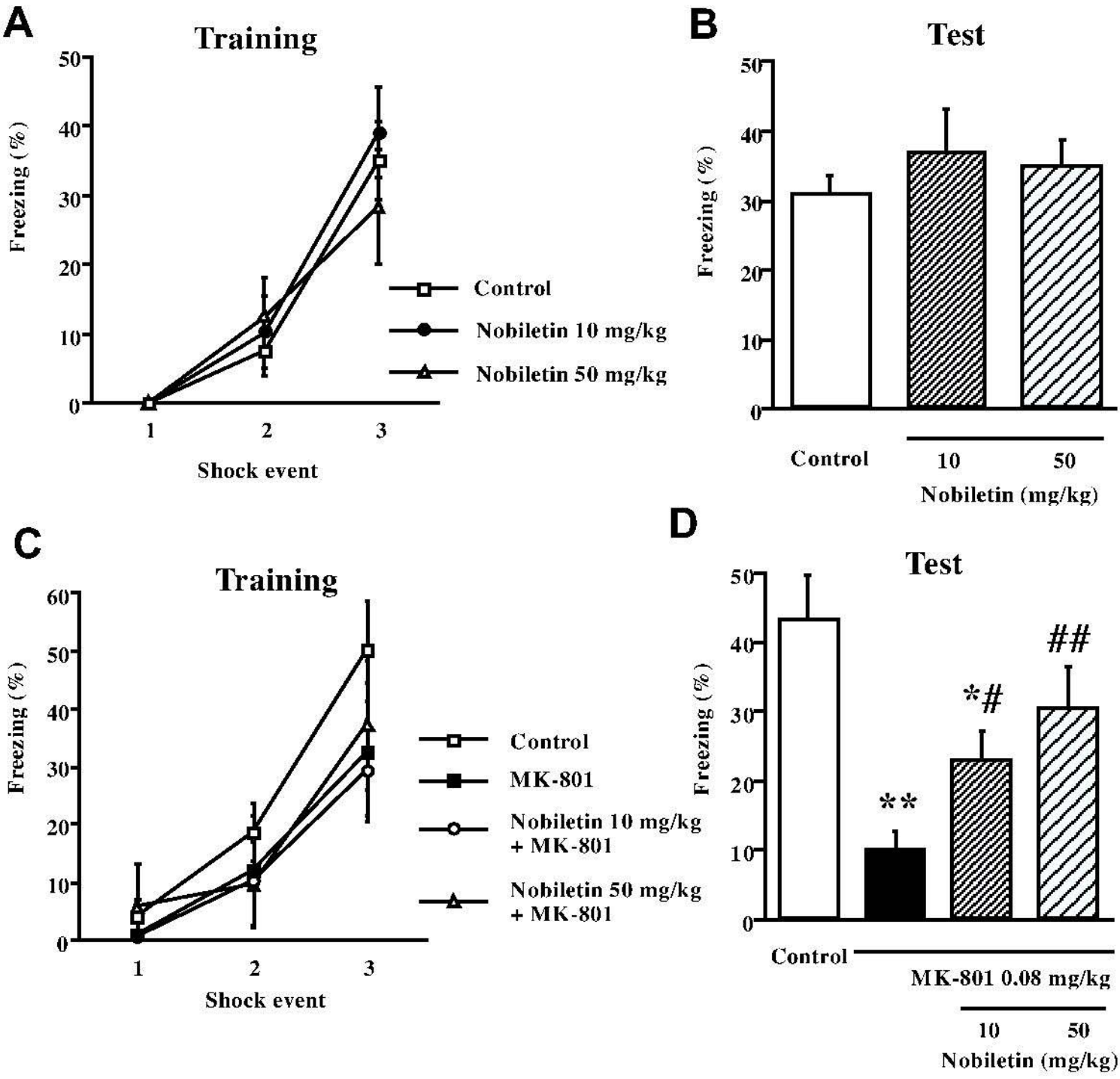

Fig. 3

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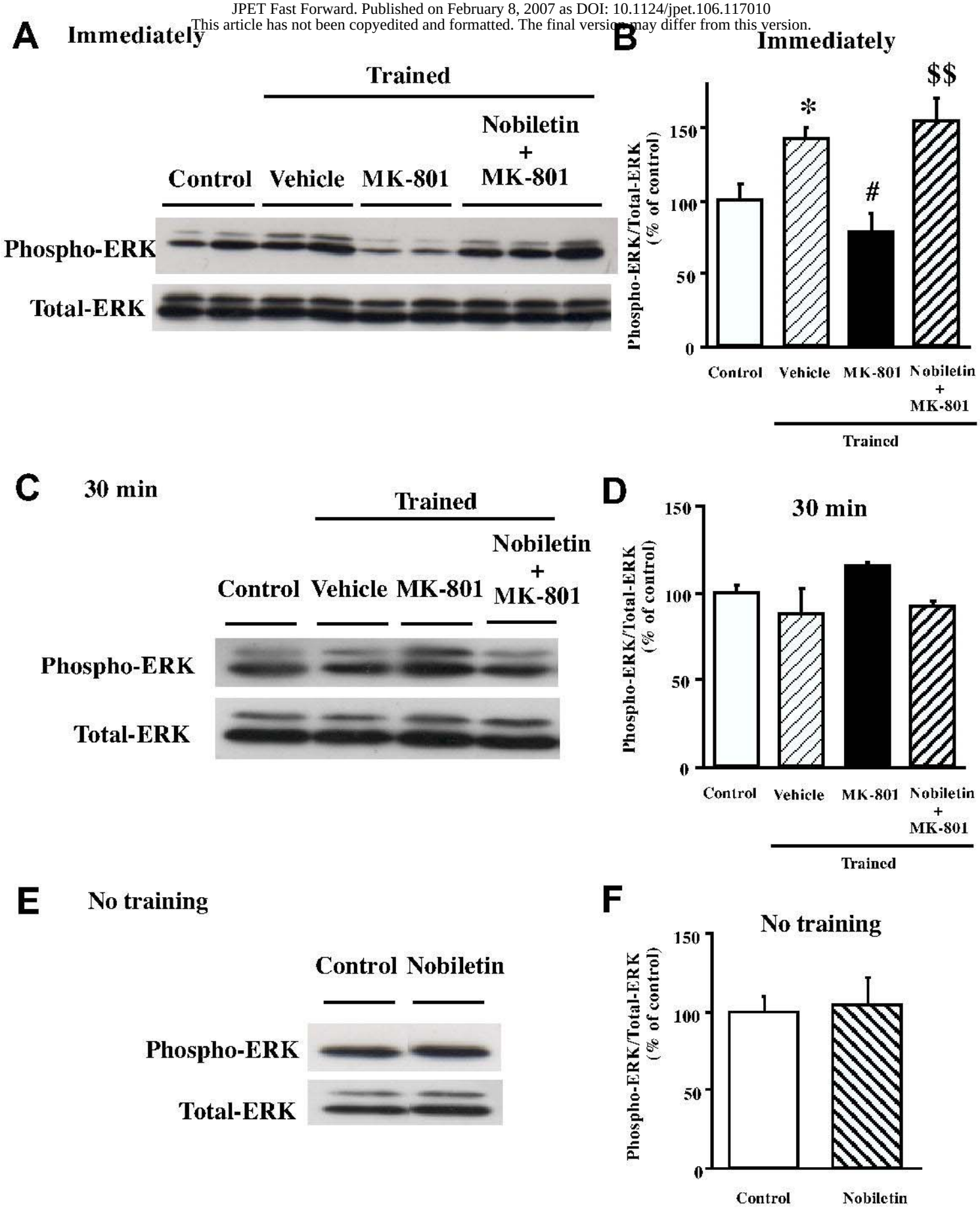

Fig. 4

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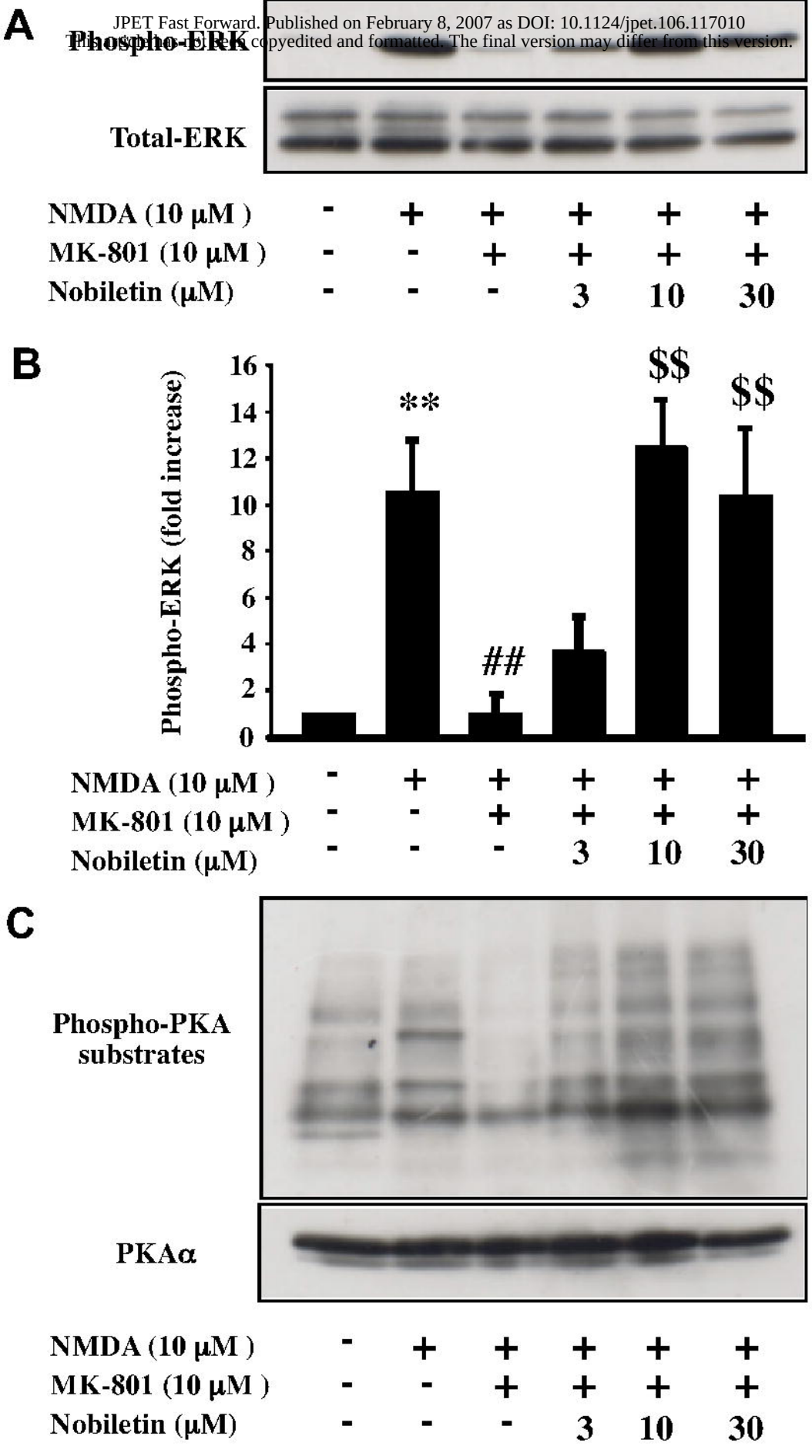

Fig. 5