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ORIGINAL PAPER

# Morphine Dependence is Attenuated by Treatment of 3,4,5-Trimethoxy Cinnamic Acid in Mice and Rats

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## Abstract

The effect of 3, 4, 5-trimethoxy cinnamic acid (TMCA) against morphine-induced dependence in mice and rats was investigated. Mice were pretreated with TMCA and then morphine was injected intraperitoneally; whereas rats were treated with TMCA (i.p.) and infused with morphine into the lateral ventricle of brain. Naloxone-induced morphine withdrawal syndrome and conditioned place preference test were performed. Moreover, western blotting and immunohistochemistry were used to measure protein expressions. Number of naloxone-precipitated jumps and conditioned place preference score in mice were attenuated by TMCA. Likewise, TMCA attenuated morphine dependent behavioral patterns such as diarrhea, grooming, penis licking, rearing, teeth chattering, and vocalization in rats. Moreover, the expression levels of pNR1and pERK in the frontal cortex of mice and cultured cortical neurons were diminished by TMCA. In the striatum, pERK expression was attenuated despite unaltered expression of pNR1 and NR1. Interestingly, morphine-induced elevations of FosB/ΔFosB$^+$ cells were suppressed by TMCA (50, 100 mg/kg) in the nucleus accumbens sub-shell region of mice. In conclusion, TMCA could be considered as potential therapeutic agent against morphine-induced dependence.

**Keywords** 3, 4, 5-Trimethoxy cinnamic acid (TMCA) · Morphine dependence · NMDA · Nucleus accumbens · FosB

## Introduction

Morphine is the usual analgesics for the treatment of sustained pain, however, patients frequently exposed to morphine develop dependence when morphine therapy is abruptly stopped. This is observed when a general opioid receptor antagonist naloxone is used after continuous opioid treatment that lead a characteristic behavior of opioid physical dependence [1]. Several pathways have been suggested for opioid dependence and analgesic effect such as the dopaminergic [2], GABAergic [3], noradrenergic [4], serotonergic [5] and glutamatergic [6] signaling cascades.

Previous studies on morphine dependence and tolerance have identified a number of signaling proteins such as *N*-methyl-D-aspartate receptor (NMDAR), nitric oxide synthase (NOS), protein kinase C (PKC), protein kinase A (PKA), calcium ($Ca^{2+}$)/calmodulin-dependent kinase II (CaMKII), delta-opioid receptor (DOR) and the regulators of G-protein signaling (RGS) proteins [7, 8]. Moreover, activation of extracellular signal-regulated kinases (ERKs) [9] and modulation of NMDAR subunit 1 (NR1) were reported in naloxone-induced morphine withdrawal [10]. This is most commonly seen in neurons of nucleus accumbens, an area rich in both opioid binding mu-opioid receptors and glutamate-binding NMDA receptors, where NR1 phosphorylation was long lasting (up to 2 months) after acute withdrawal [11].

FosB-like (FosB/ΔFosB) proteins are encoded in early-gene Fos family that play critical roles in neuronal regulations after repeated drug administration [12]. Opioid-induced expression of FosB/ΔFosB is considered as a biomarker of specific neural plasticity for repeated exposure [13]. This has been reported in the use of cannabinoids, cocaine, or morphine associated with increased FosB/ΔFosB levels in prefrontal cortex, nucleus accumbens and dorsal striatum, which are brain regions related to positive reinforcing effects of drugs [14].

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Methoxycinnamic acids are important substances for the remediation of serious diseases such as oncology [15]. In addition, they have been used as anti-inflammatory [16], and anti-enzyme agents [17]. A previous report by Kawashima group [18] indicated that 3,4,5-trimethoxy cinnamic acids (TMCAs) have shown anti-stress effects via suppressing norepinephrine (NE) content in the brain region of locus coeruleus, which has been known for elevated content of NE during morphine withdrawal syndrome.

Considering our study on medicinal chemistry that deals with TMCA and its derivatives with remarkable bioactivities for neurological disorders, we have shown the role of 3, 4, 5-trimethoxyphenyl acrylamides and TMCA derivatives with good antinarcotic effect in mice due to their high binding affinities with serotonergic 5-$HT1_A$ receptors [19, 20]. However, little is known regarding to TMCA derivatives that can enable to attenuate naloxone-induced behavioral patterns in morphine dependent mice. Moreover, the main target of TMCA on neurotransmitter pathways and receptors in the central nervous system (CNS) have not been unraveled. Here, therefore, we clearly showed that TMCA attenuated morphine-induced dependence, and reduced expression of pNR1 and pERK in the frontal cortex and ΔFosB in the nucleus accumbens shell region of mice.

## Materials and Methods

### Drugs

Morphine hydrochloride (Myungmun Pharm, Seoul, Korea) and naloxone hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in physiological saline. TMCA (Sigma-Aldrich, St. Louis, MO, USA) was prepared using 10% cremophor (Sigma-Aldrich, St. Louis, MO, USA) solution containing 2% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA). All drugs were freshly prepared and injected into mice.

### Animals and Treatments

C57BL/6 male mice and male Sprague–Dawley rats both were obtained from Daehan Biolink (Eumsung, Korea). Animals were housed and acclimatized (1 week) on a 12 h light–dark cycle and maintained at 24 ± 3 °C with free access to chow and water. All animal procedures were in accordance with the Institutional Animal Care and Use Committee of Ewha Womans University, School of Medicine.

Mice (male, 20 ± 2 g) were randomly divided into each group and were given saline, morphine, or both morphine and TMCA. Morphine chloride (10 mg/kg/day) was dissolved in saline whereas TMCA (10–100 mg/kg/day) was prepared using cremophor and DMSO. Mice were pretreated with TMCA for 30 min before using morphine, both of which were administered intraperitoneally daily for 7 days. Finally, naloxone hydrochloride (5 or 10 mg/kg, i.p.) was injected 6 h after final morphine treatment in mice thereby induction of morphine withdrawal syndrome.

Drug infusion into brain of rats (220–240 g) was performed as described previously [21]. Briefly, rats were anesthetized by ketamine (50 mg/kg) and xylazine (1 mg/kg) before surgery. A 21-gauge stainless steel cannula was implanted in the right lateral ventricle of rat brain. Rats were allowed 1 week for recovery. Then, a mini-pump was implanted subcutaneously. After a small cut was made behind the ears of the rat, saline vehicle or TMCA was filtered through a 0.2 μm filter to fill an osmotic mini-pump (Alzet 2ML 1, Alza, Palo Alto, CA, USA). The mini-pump which contains saline or morphine was implanted and connected directly to the cannula via 6-cm long PE-60 polyethylene tubing. Then, morphine was infused at the rate of 26 nmol/10μ l/h for 7 days.

### Measurement of Morphine Withdrawal Syndrome

Mice were injected with naloxone (5 mg/kg) and then immediately they were placed into individual observation acryl cylinders (25 cm in diameter and 50 cm in high). Next, jumping frequencies of each mouse were observed for 30 min. Video camera was set and behavior was recorded for all experiments by an observer who was blind to details of experiment. In rats, 6 h after termination of morphine infusion, naloxone (10 mg/kg, i.p.) was injected and then rats were kept in plastic rat cages for 30 min to observe withdrawal symptoms like wet-dog shake, escape behavior, grooming, and teeth chattering.

### Conditioned Place Preference Test

Conditioned place preference (CPP) test was used to evaluate morphine dependence in mice as described before [22]. Briefly, each mouse was individually placed into plexiglass apparatus for 15 min at day 1 (pre-conditioning). Then, guillotine doors were raised to allow mice for free access to both compartments. On Day 2, the time spent in each chamber was recorded for 15 min and analyzed. During the conditioning phase, half of the mice in each group were confined to the black compartment, and the other half were confined to the white compartment every day for six consecutive days. On day 3, 5, and 7, mice received drug just prior to being confined in the compartment that they less preferred for 30 min. On day 4, 6, and 8, mice received saline just prior to being confined to the compartment they favored for 30 min. During the post-conditioning phase (Day 9), the guillotine doors were raised, and the untreated mice were placed in the tunnel in the central part of the apparatus. The time that mice

spent in each compartment was recorded for 15 min. CPP scores were expressed as the difference in the preconditioning and post-conditioning. All experiments were carried out between 2:00 and 5:00 p.m.

### Primary Cortical Culture

Cortical cell culture was prepared from C57BL/6 mice embryo at gestational age of 15 days. The brain was dissected; cortical tissues were taken out and then dissociated in ice-cold buffer to prepare cell suspension, which was centrifuged at 1000 rpm for 5 min, and then cell pellets were re-suspended in MEM (Gibco, BRL. Rockville, MD, USA) supplemented with 5% heat-inactivated fetal calf serum (Gibco, USA), the cells were cultured in an incubator at 5% $CO_2$ at 37 °C for 7 days. Then, cells were treated with 10 $\mu M$ cytosine arabinofuranoside (Ara C, Sigma-Aldrich, MO, USA) to reduce the growth of contaminating non-neuronal cells. Cortical neuronal cells were cultured in MEM containing 10% horse serum without glutamine. Cells were pretreated with TMCA (1, 10, 100 μM) for 30 min and then exposed to morphine (1 μM) for 6 h. Protein expression of p-NR1, NR1, p-ERK; ERK; p-CREB and CREB were examined using western blot analysis.

### Western Immunoblotting

After TMCA treatment and final morphine administration, on the 7th day, frontal cortex and striatum were collected 6 h later and protein was extracted. Protein concentration in whole cell lysates (after cortical culture) or brain tissues were determined using a protein assay kit (Pierce Biotechnology, Rockford, USA). Sample buffer (4×) was added to the protein and then it was boiled for 15 min at 100 °C. Proteins (30–60 μg) underwent electrophoresis on 10 or 8% SDS–polyacrylamide gel and then transferred to polyvinylidene difluoride membrane. The blots were then blocked with 5% fat-free dry milk-TBST (Tris-buffered saline containing 0.1% Tween-20) buffer for 1 h and incubated with primary antibodies (ERK1/2, 1:1000; pERK1/2, 1:2000; NR1, 1:1000; pNR1, 1:1000; pCREB and CREB, 1:1000; FosB and ΔFosB, 1:1000; β-actin, 1:1000; GAPDH, 1:1000, Santa Cruz Biotechnology, CA, USA) at 4 °C overnight. After washing three times with TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000) in 3% fat-free dry milk-TBS for 1 h at room temperature. The blots were rinsed again three times with TBST, and the transferred proteins were incubated with the ECL chemiluminescent detection kit according to the manufacturer's instructions and visualized with image-quant™ LAS 4000 (Fujifilm Life Science, Tokyo, Japan).

### Immunohistochemistry

Mice were perfused through the left cardiac ventricle with 10 mL of 10 mM phosphate buffer (pH 7.4) followed by 40 mL of a cold fixative (4% paraformaldehyde in 100 mM phosphate buffer). After perfusion, the brain tissues were quickly removed, post-fixed for 18 h with the same fixative at 4 °C, and transferred to 10%, then 20%, and then 30% sucrose solution. Sections (40 μm thick) were prepared using a vibratome (Leica, Wetzlar, Germany).

Free-floating sections were treated with 0.3% Triton-X 100 for 30 min, and non-specific protein binding was blocked by incubation with 10% normal serum in PBS for 1 h. Sections were incubated with anti-FosB antibody at 4 °C overnight. Primary antibody was reacted with biotinylated secondary antibody in PBS blocking buffer and visualized by the ABC method (ABC Elite kit, Vector Lab., Burlingame, CA, USA). Sections were mounted, and images were analyzed using an Olympus BX 51 microscope equipped with a DP71 camera and DP-B software (Olympus Co., Tokyo, Japan). The number of anti-FosB/ΔFosB positive cells were assessed using digital images (captured at 100× magnification) using the Image J program (NIH Image Engineering, Bethesda, MD).

### Statistical Analysis

All values were expressed as mean ± standard deviation (SD). Results were subjected to one-way ANOVA followed by the Newman-Keuls multiple comparison test. Differences with $*p < 0.05$, $**p < 0.01$, were considered statistically significant.

## Results

### TMCA Reduced Morphine-Induced Physical Dependence in Mice

Long term treatment of morphine in mice is associated with physical dependence, which was frequently observed with jumping behavior immediately after injection of naloxone (5 mg/kg). To determine whether TMCA (10–100 mg/kg) pretreatment for 7 days would have mitigated physical dependence of morphine injection (10 mg/kg, i.p.), individual mouse was kept separately for 30 min using plastic cylinders. It was found that morphine-treated mice showed the high frequency of jumping after injection of naloxone. However, the number of jumps were reduced by threefold and sixfold when TMCA was used at a concentration of 50 mg/kg and 100 mg/kg, respectively. Treatment of mice

using TMCA alone at the highest concentration (100 mg/kg) showed comparable findings with that of saline-treated mice (Fig. 1).

## Conditioned Place Preference (CPP) was Attenuated by TMCA in Mice

CPP is the usual sequel when mice is subjected to extended period of morphine treatment. To determine if mice pretreated with TMCA and then injected with morphine were less likely to develop psychological dependence, the CPP test was used. Morphine (10 mg/kg, i.p.) and TMCA (50, 100 mg/kg) were administered daily for 7 days intraperitoneally. Scoring results of conditioned place preference test indicated that mice treated with morphine alone showed an increment; however, this was remarkably reduced by TMCA at the dose of 100 mg/kg (Fig. 2).

## Withdrawal Symptoms of Morphine was Inhibited by TMCA Treatment in Rats

We also opted to determine the action of TMCA against morphine dependence in rats. Here, we used TMCA at100 mg/kg (i.p.) in rats infused with morphine (26 nmol/10 ul/h, i.c.v) into the right lateral ventricle of brain. Behavioral patterns such as diarrhea, penis licking, teeth chattering and vocalization were markedly reduced by TMCA pretreatment

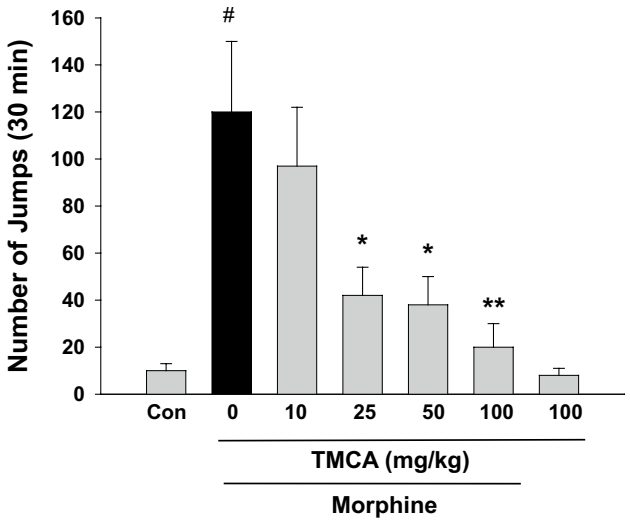


**Fig. 1** Effects of TMCA on naloxone-precipitated jumping behavior in morphine-dependent mice. TMCA (10, 25, 50, 100 mg/kg, i.p.) was administered 30 min before morphine injection (10 mg/kg, i.p., n = 10) for 7 days. On the 7th day, naloxone (5 mg/kg, i.p.) was injected 6 h after final morphine administration and then jumping frequencies in 30 min were counted. Data were expressed as mean ± SD. (n = 10). #p < 0.01 in comparison with control, *p < 0.05, **p < 0.01 in comparison with saline (morphine only) group, one-way ANOVA followed by the Newman-Keuls test

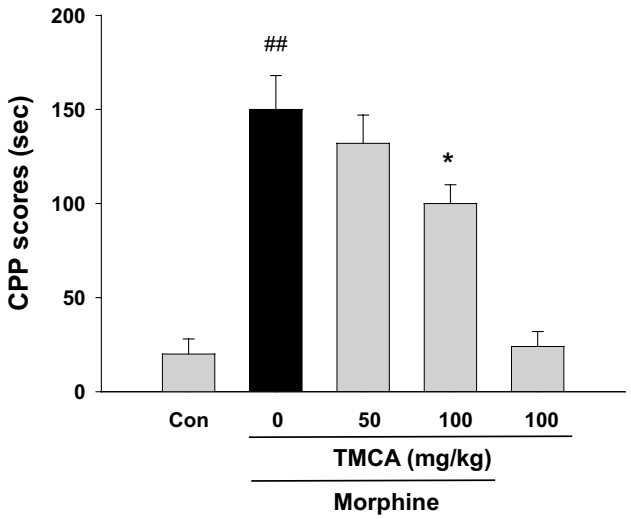


**Fig. 2** The effect of TMCA pretreatment against morphine-induced CPP in mice. TMCA (50, 100 mg/kg, i.p) and morphine (10 mg/kg, i.p) were used in mice. The time that mice spent in each compartment of the CPP apparatus was recorded for 15 min. The CPP scores were calculated by taking the difference between preconditioning and post-conditioning. Data were expressed as mean ± SD. (n = 8). ##p < 0.01 compared to the control group (each group, n = 8); *p < 0.05 compared to the morphine-only group, one-way ANOVA followed by the Newman-Keuls test

(Fig. 3a, d, f, g). Although behavioral patterns like grooming and rearing were moderately reduced by pretreatment of TMCA, escape behavior and wet-dog shake symptoms were unchanged (Fig. 3b, h).

## Effect of TMCA on Phosphorylated NR1 and ERK1/2 in Mice Brain and Cultured Cortical Neurons

Morphine induces activation of extracellular signal-regulated kinases and NMDAR subunit 1, which are intricately interacting with G protein-coupled μ-opioid receptors leading to morphine associated dependence and tolerance [9]. Therefore, we determined if morphine-related activation of phospho-NR1 and phospho-ERK expression in the frontal cortex of mice and cultured cortical cells were modulated by TMCA. We found that TMCA (50 and 100 mg/kg) attenuated p-ERK, p-NR1 and NR1 protein expressions in the frontal cortex of mice brain. On the contrary, TMCA (100 mg/kg) alone induced elevation of pERK expression in the cortex of mice (Fig. 4a). However, neither pNR1 nor NR1 was modulated by morphine or TMCA treatment in the striatum, (Fig. 4b). Instead, pERK expression was increased by morphine treatment and this was inhibited by treatment with TMCA 100 mg/kg (i.p.). Under in vitro conditions, protein expression of p-NR1, total NR1, pERK and pCREB expressions were elevated in cultured cortical neurons subjected to

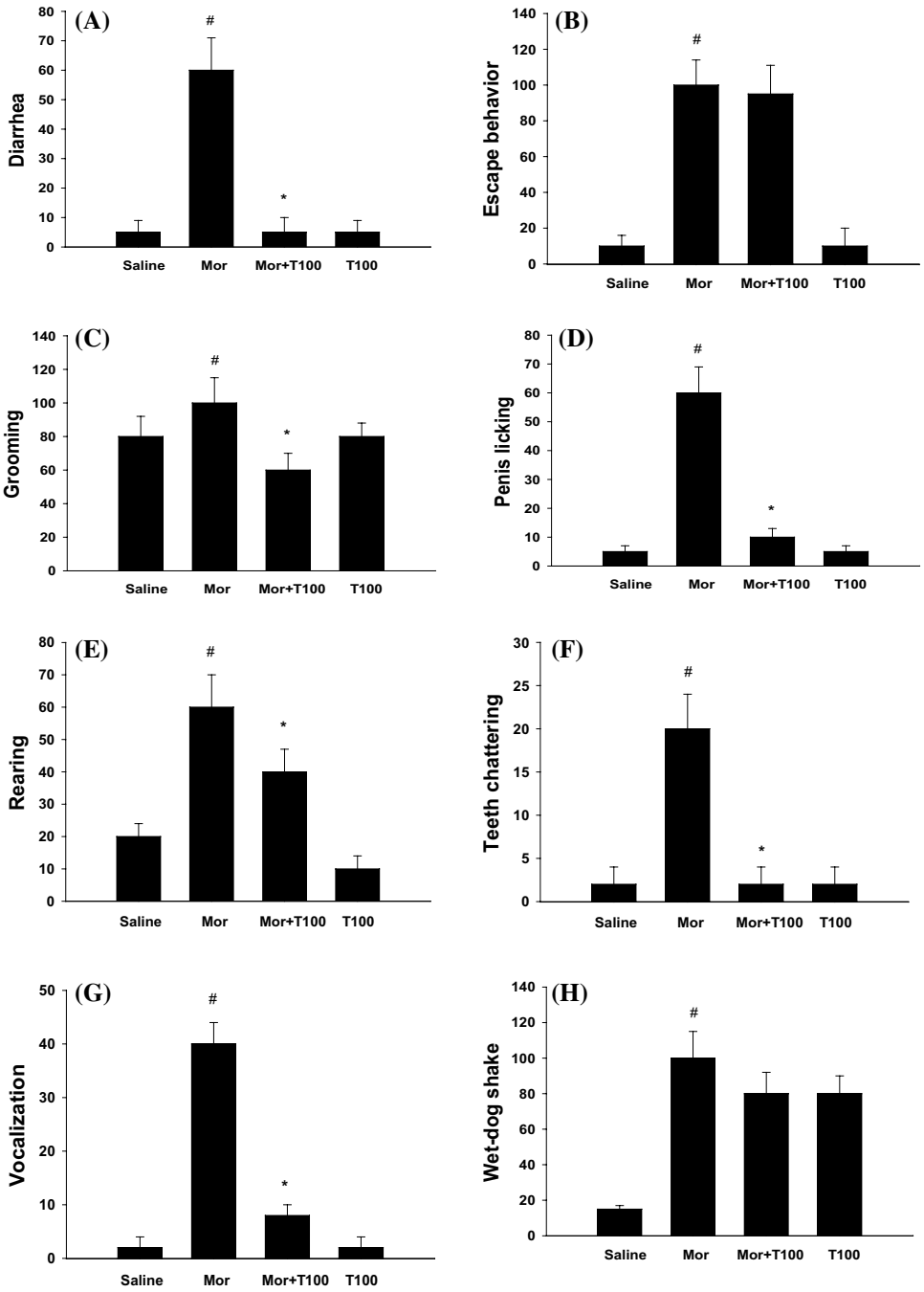


**Fig. 3** Inhibitory effects of TMCA on morphine withdrawal signs in rats. Rats were infused with morphine (26 nmol/10 µl/h) and/or TMCA was administered (10 mg/kg, i.p.) for 7 days, and then treated with naloxone (10 mg/kg, i.p.) 6 h after the cessation of morphine infusion. Withdrawal signs were observed for 30 min after injection of naloxone. Data were expressed as mean ± SD. (n = 6) #p < 0.05 in comparison with control (saline), *p < 0.05, in comparison with morphine alone group, one-way ANOVA followed by the Newman-Keuls test

morphine; however, these were suppressed by treatment of TMCA at higher concentration (100 µM) (Fig. 5).

## Effect of TMCA on the Expression of ΔFosB in the Nucleus Accumbens of Mice

Given that TMCA was able to mitigate morphine withdrawal behavior, we made an attempt to identify the effects of TMCA on ΔFosB induction in nucleus accumbens (NAc) shell region. It was clear that ΔFosB protein expression in the NAc shell region was markedly increased by morphine treatment (10 mg/kg, i.p.) unlike that of FosB (Fig. 6a). Likewise, FosB/ΔFosB$^+$ cell numbers were profoundly elevated during morphine treatment (Fig. 6b); however, mice pretreated with TMCA (25 and 50 mg/kg) revealed a reduced FosB/ΔFosB$^+$ cell numbers in the NAc shell region, although no alteration of these cells were detected in the NAc core region (Fig. 6b, c).

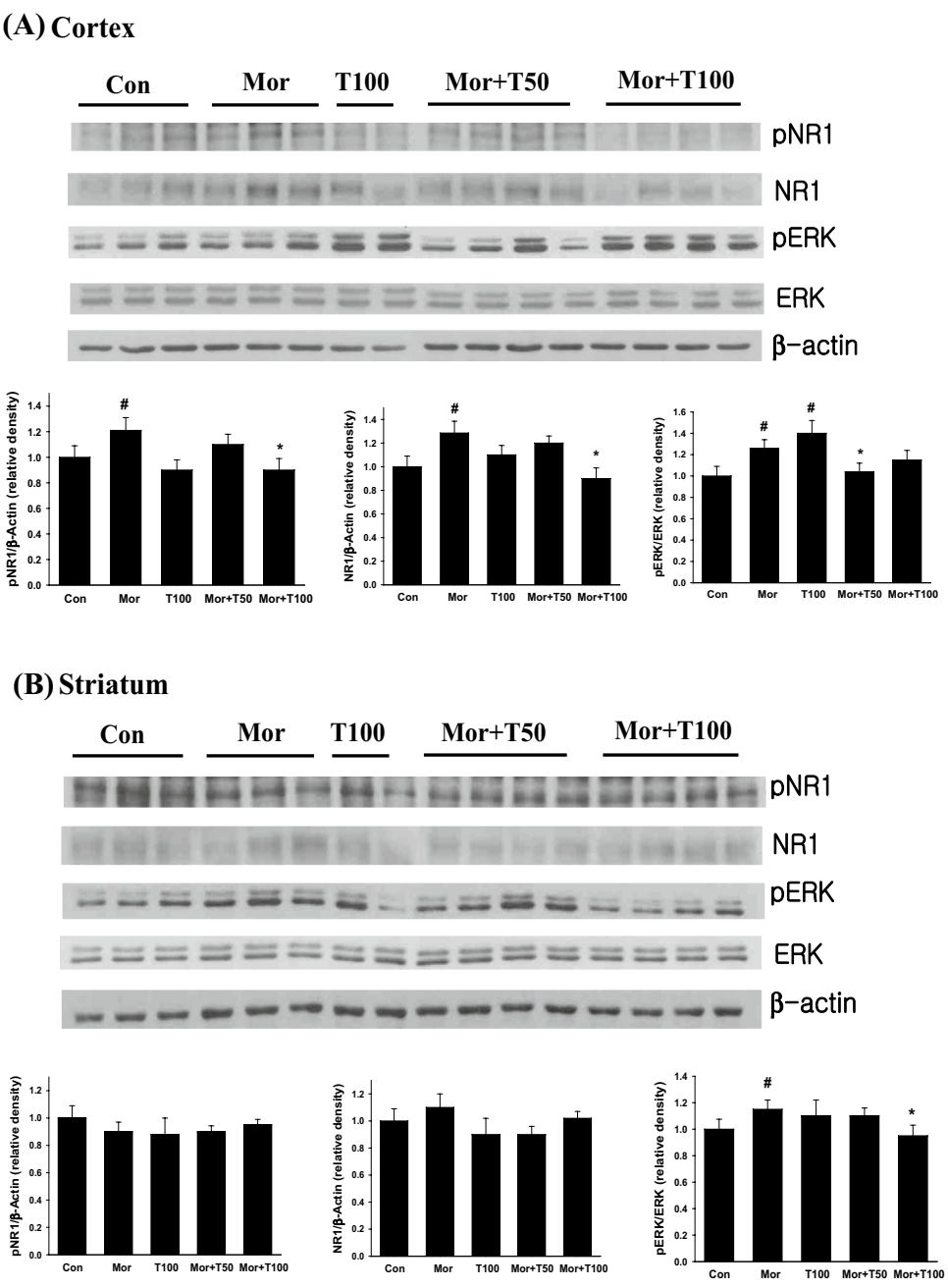


**Fig. 4** Effects of TMCA on morphine-induced phosphorylation of NR1 and ERK in the frontal cortex (**A**) or striatum (**B**) region of mice. TMCA (50, 100 mg/kg, i.p.) were administered to mice 30 min before morphine injection (10 mg/kg, i.p.) for 7 days. On the 7th day, frontal cortex and striatum were collected 6 h after final morphine administration. Protein expressions of p-NR1, NR1, p-ERK, and ERK were examined using western blot analysis. Data were expressed as mean ± SD. (n = 6) $^{\#}P < 0.05$, compared with control (vehicle) group. $^{*}P < 0.05$, compared with the morphine alone group, one-way ANOVA followed by the Newman-Keuls test

## Discussion

The use of morphine for chronic pain management has been inevitable despite its negative consequences such as dependence and tolerance among others. A recent finding also revealed that gut microbiome and metabolome as a potential mechanism contributing to these negative consequences associated with morphine use [23]. Considering the unwanted effects of long term morphine therapy, we have been looking for potential candidate compounds that can mitigate morphine dependence and tolerance [21, 22, 24]. Looking into 3, 4, 5-trimethoxy cinnamic acid (TMCA) that can ameliorate restraint stress-provoked anxiety- and depression-like behaviors in mice [25], we questioned that TMCA and its derivatives could be applied to reduce morphine dependence and tolerance. We found that TMCA diminished the number of naloxone-induced jumps and CPP scores in mice. Similarly, behavioral patterns (diarrhea, penis licking, teeth chattering and vocalization) of rats associated with long term use of morphine were markedly attenuated. Together, these findings were in line with our previous work on ginsenosides working against morphine dependence [21]. However, escape behavior and wet-dog shake symptoms in rats were unaltered by TMCA treatment, which may indicate

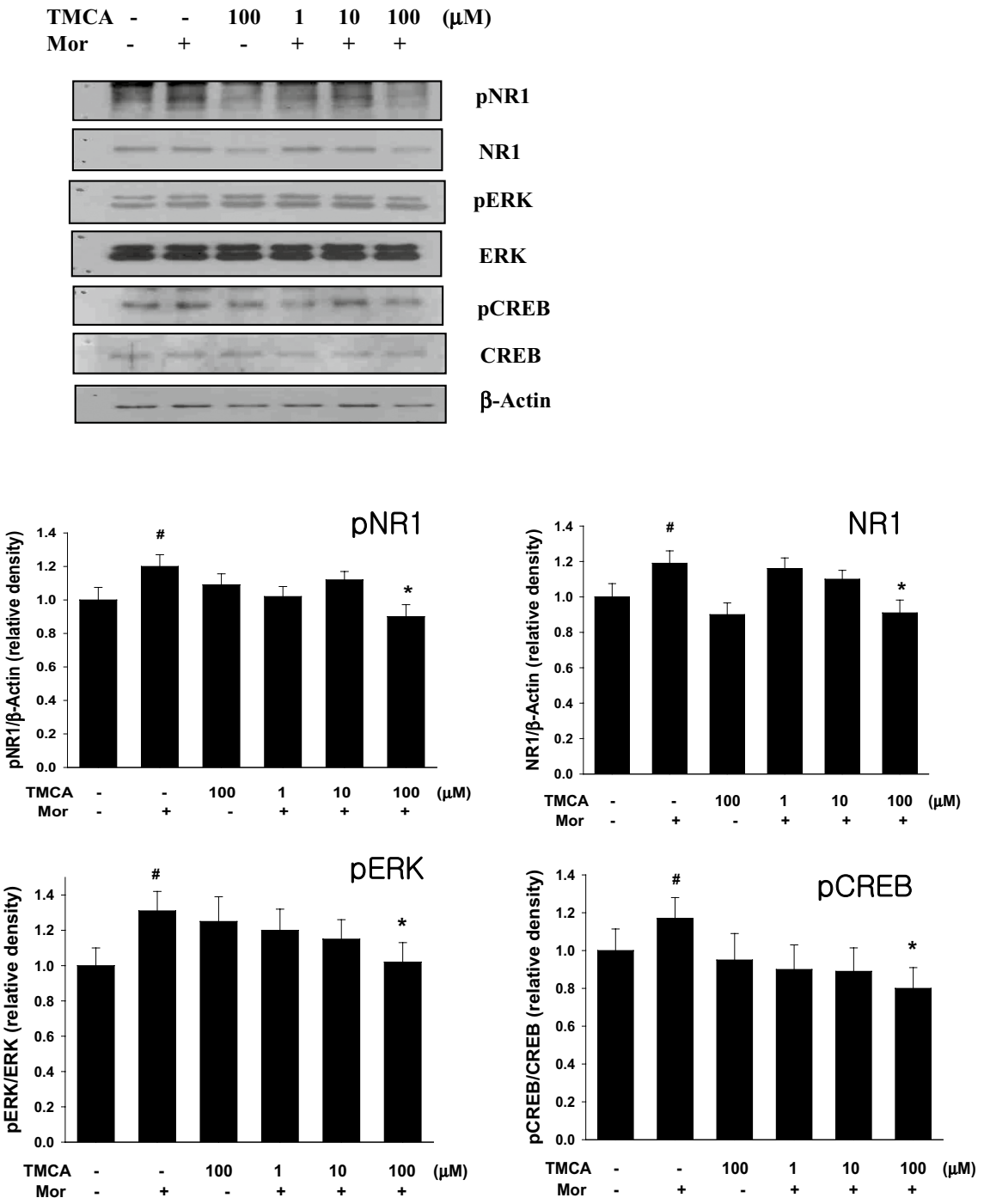


**Fig. 5** Changes in protein expressions of NR1, NR1; pERK, ERK; pCREB and CREB were measured in cultured cortical neurons. Cells were pretreated with TMCA (1, 10, 100 μM) for 30 min and then exposed to morphine (1 μM) for 6 h. The expression levels of aforementioned proteins were examined using western blot analysis. All values were expressed as mean ± SD from three independent experiments. $^{\#}P < 0.05$, compared with vehicle (not treated with TMCA or morphine). $^{*}P < 0.05$, compared with the morphine group, one-way ANOVA followed by the Newman-Keuls test

its preferential effect on behavioral patterns in rats exposed to morphine.

Chronic morphine therapy has been linked with increased expression of connexin 43 (Cx43) and NMDAR subunit 1 (NR1), which are associated with deleterious effects such as dependence and tolerance [26]. One of the molecular mechanisms associated with these unwanted effects is the NMDA-receptor-ERK signaling pathway

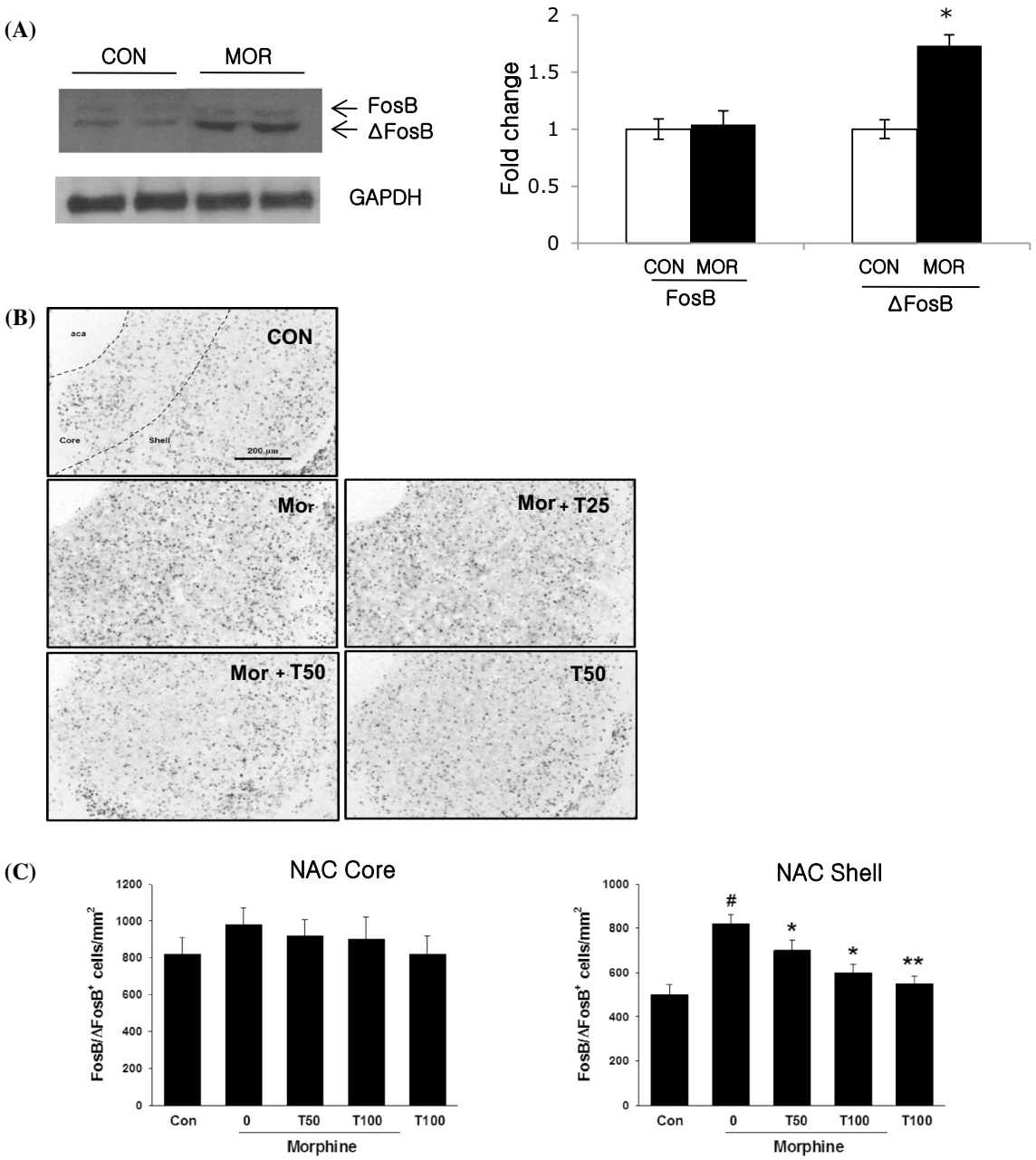


**Fig. 6** Long-lasting stimulation of ΔFosB in nucleus accumbens in morphine-treated mice was suppressed by TMCA. (**A**) Western blot analysis showing the immunoreactivity against the anti-FosB/ΔFosB antibody and quantitative analysis of western blots; (**B**) photomicrographs showing anti-ΔFosB immunoreactivity in nucleus accumbens regions, and (**C**) quantitative analysis of immunohistochemistry. Data were presented as mean ± SEM. $^{\#}P<0.05$, compared with control (vehicle) group. $^{*}P<0.05$, compared with the morphine alone group, one-way ANOVA followed by the Newman-Keuls test

[27]. We found that TMCA attenuated protein expression of pNR1, NR1 and pERK induced by morphine in the frontal cortex of mice. In addition, expression of pCREB protein was downregulated by TMCA in cultured cortical neurons. In the striatum, pERK expression was attenuated despite unaltered expression of pNR1 and NR1. It seemed that the action of TMCA on NMDA-receptor-ERK signaling vary depending of the brain regions. Such kind of selective action on one signaling protein over the other was also observed during our report on ginsenosides working against morphine dependence [21]. Although it is difficult to forward a concreate explanation at this stage, the physiological status of mice during daily delivery of drugs may account for these conflicting results.

ΔFosB (one of Fos-family transcription factors) is unique since it accumulates in response to repeated stimulation due to its unusual protein stability. ΔFosB within nucleus accumbens (NAc), a key brain reward region, has been most studied in animal models of drug addiction [13]. Our study within nucleus accumbens region of the brain indicated that ΔFosB protein expression was increased when mice exposed to morphine (Fig. 6a). In the NAc shell region, TMCA attenuated the expression of ΔFosB as it was revealed by a decrease in FosB/ΔFosB$^+$ cells per mm$^2$. This finding is consistent with a decreased FosB/ΔFosB levels found in morphine dependent mice after treatment with agmatine [28]. However, whether TMCA share a similar mechanism of action with agmatine or follow a different one needs further investigation.

In conclusion, we demonstrated that TMCA attenuated morphine-induced physical and psychological dependence, which could be mediated by the activation of NMDA receptor-ERK signaling pathway. Moreover, morphine-induced elevations of ΔFosB positive cells in NAc shell regions were diminished by TMCA. It seems that ΔFosB is the downstream target of TMCA under NMDA receptor-ERK signaling pathway, however; this warrants further investigation. TMCA could be a potential therapeutic agent to ameliorate the morphine dependence.

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