

# Haloperidol Disrupts Opioid-Antinociceptive Tolerance and Physical Dependence

Cheng Yang, Yan Chen, Lei Tang, and Zaijie Jim Wang

*Department of Biopharmaceutical Sciences and Cancer Center, University of Illinois, Chicago, Illinois*

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## ABSTRACT

Previous studies from our laboratory and others have implicated a critical role of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) in opioid tolerance and dependence. Translational research targeting the CaMKII pathway is challenging, if not impossible, because of a lack of selective inhibitors. We discovered in a preliminary study that haloperidol, a butyrophenone antipsychotic drug, inhibited CaMKII, which led us to hypothesize that haloperidol can attenuate opioid tolerance and dependence by inhibiting CaMKII. The hypothesis was tested in two rodent models of opioid tolerance and dependence. Pretreatment with haloperidol (0.2–1.0 mg/kg i.p.) prevented the development of morphine tolerance and dependence in a dose-dependent manner. Short-term treatment with

haloperidol (0.06–0.60 mg/kg i.p.) dose-dependently reversed the established morphine-antinociceptive tolerance and physical dependence. Correlating with behavioral effects, pretreatment or short-term treatment with haloperidol dose-dependently inhibited morphine-induced up-regulation of supraspinal and spinal CaMKII $\alpha$  activity. Moreover, haloperidol given orally was also effective in attenuating morphine-induced CaMKII $\alpha$  activity, antinociceptive tolerance, and physical dependence. Taken together, these data suggest that haloperidol attenuates opioid tolerance and dependence by suppressing CaMKII activity. Because haloperidol is a clinically used drug that can be taken orally, we propose that the drug may be of use in attenuating opioid tolerance and dependence.

## Introduction

Opioids are highly efficacious analgesic drugs. However, repeated use of these drugs leads to the development of tolerance and dependence, thereby limiting their effectiveness and usage. The mechanisms underlying opioid tolerance and dependence are not entirely understood. Studies from our laboratory and others have begun to unravel a critical role of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) in opioid tolerance and dependence (Wang and Wang, 2006). CaMKII is a multifunctional, Ca<sup>2+</sup>/calmodulin-activated protein kinase that was originally discovered in the brain (Schulman and Greengard, 1978). It has since been shown to be a critical mediator of neuronal plasticity and play a key role in long-term potentiation, learning and memory (Lee, 2006; Wayman et al., 2008; Redondo et al., 2010). Long-term treatment with morphine has been shown to increase CaMKII activity in vivo (Wang et al., 2003; Liang et al., 2004; Tang et

al., 2006a). Supraspinal and spinal inhibition of CaMKII not only prevented but also reversed opioid-antinociceptive tolerance and physical dependence in several rodent models (Wang et al., 2003; Tang et al., 2006a). These data support a critical role of CaMKII in the development and maintenance of opioid tolerance and dependence. Furthermore, inhibiting CaMKII $\alpha$  by chemical inhibitors, small interfering RNA, and gene deletion methods attenuated opioid-induced hyperalgesia, a clinical and experimental phenomenon that is highly relevant for tolerance (Chen et al., 2010). Therefore, targeting CaMKII or its signaling pathways may provide potential targets of pharmacological intervention for alleviating opioid tolerance or dependence.

Searching for selective chemical inhibitors of CaMKII has not been very successful, because it is difficult to specifically inhibit a protein kinase without affecting a closely related isoform. Here, we have focused our efforts on clinically used drugs that may inhibit CaMKII. Haloperidol belongs to the typical antipsychotic drug class. These drugs are thought to block dopamine D2 receptors, although, similar to most central nervous system drugs, the exact mechanism of action is not entirely understood. The interactions between the dopamine and opioid systems have been studied extensively (e.g., Unterwald and Cuntapay, 2000). In fact, many of these

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**ABBREVIATIONS:** CaMKII, Ca<sup>2+</sup>/calmodulin-dependent protein kinase II; NMDA, *N*-methyl-D-aspartate;  $\mu$ OR, the  $\mu$ -opioid receptor; MPE, maximal possible effect; MK801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate.

studies have used typical antipsychotic drugs to block the dopamine activity. However, these drugs also may have other actions (Tang et al., 2006b; Chen et al., 2009). In this study, we tested the hypothesis that haloperidol can inhibit CaMKII and attenuate opioid-antinociceptive tolerance and physical dependence in two rodent models.

## Materials and Methods

Morphine sulfate was provided by the National Institutes of Health National Institute on Drug Abuse (Bethesda, MD). Haloperidol, naloxone, and other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO). Male ICR mice ( $25 \pm 5$  g; Harlan Laboratories, Indianapolis, IN) were kept on a 14/10-h light/darkness cycle (5:00 AM on and 7:00 PM off) and provided food and water ad libitum before experimental procedures. Mice were randomly divided into experimental groups according to a computer-generated randomization list. Behavioral tests were performed by an experimenter blinded to specific group and treatment information. All experiments were performed in accordance with the policies and recommendations of the National Institutes of Health guidelines for the handling and use of laboratory animals after approval by the University of Illinois Institutional Animal Care and Use Committee.

**Tail-Flick Test.** The tail-flick test was used to determine basal nociception and morphine antinociception as described previously (Wang et al., 2001; Tang et al., 2006a). In brief, One third of the distal portion of mouse tail was immersed into a water bath maintained at 52°C, and the latency of a quick tail-flick response was recorded. Morphine-antinociception was evaluated 30 min after a test dose of morphine (10 mg/kg s.c. unless otherwise stated) and is expressed as the percentage of maximal possible effect (MPE).  $MPE\% = 100\% \times (\text{post-drug latency} - \text{predrug latency})/(\text{cutoff} - \text{predrug latency})$ . A 12-s cutoff time was used to prevent tissue damage.

**Acute Opioid Tolerance and Dependence.** To induce acute opioid tolerance and dependence, mice were treated with morphine sulfate (100 mg/kg s.c., time 0) (Yano and Takemori, 1977; Bilsky et al., 1996; Tang et al., 2006a). Morphine tolerance and dependence developed within hours and peaked at approximately 4 to 6 h (Shukla et al., 2006). Control mice received an equal volume of saline. Tolerance was assessed by monitoring reduced antinociception of a test dose of morphine (10 mg/kg s.c., given at 4.5 h) by use of the tail-flick test. Before the injection of the test dose of morphine, baseline tail-flick latency was re-established. In all mice, tail-flick latencies had returned to normal values at that time. To examine opioid dependence, morphine- or saline-pretreated mice were challenged with naloxone (10 mg/kg i.p.). Mice were immediately placed into glass cylinders, and the number of vertical jumps was recorded for 15 min (Tang et al., 2006a). The presence of the physical dependence of morphine was indicated by a significant number of naloxone-precipitated jumps compared with that of saline-treated mice.

To prevent morphine tolerance and dependence, haloperidol (0.06–0.60 mg/kg i.p.) was given 30 min before the induction dose of morphine (100 mg/kg s.c.). To reverse morphine tolerance and dependence, haloperidol (0.06–0.60 mg/kg i.p.) was given 30 min before the test dose of morphine (10 mg/kg) or naloxone.

**Chronic Opioid Tolerance and Dependence.** To induce chronic opioid tolerance and dependence, mice were treated with morphine (10 mg/kg s.c., given at 8:00 AM and 6:00 PM) for 5 days (Herz and Teschemacher, 1973). Control mice received an equal number and volume of injections with saline. Morphine tolerance and naloxone-precipitated withdrawal were evaluated as described above. Haloperidol (0.1–1.0 mg/kg i.p. or p.o.) was given 30 min before the test dose of morphine or naloxone.

**Rotarod Test.** To examine whether haloperidol may cause locomotor impairment, experiments using a rotarod test were conducted as described previously (Chen et al., 2010). Mice were placed on a

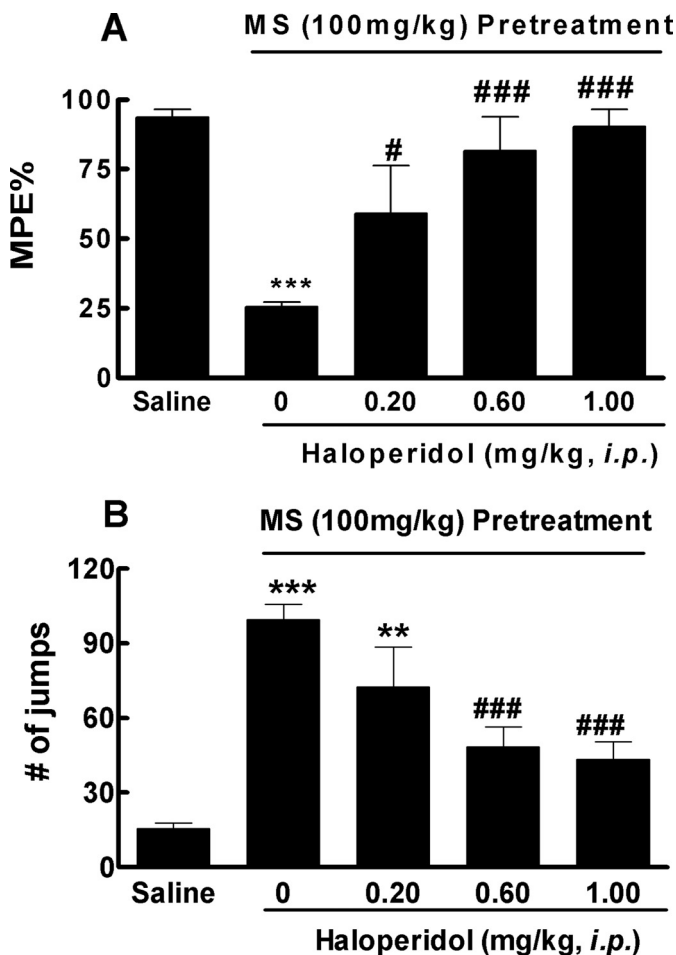
1.25 inch-diameter rod powered by a motor with adjustable speeds (Model series 8; IITC, Woodland Hills, CA). On day 1, mice were trained to remain on a fixed speed (4 rpm) for 60s. On the next day, mice were retrained at the same speed. Those mice that failed to stay on the rotarod for 60 s were eliminated from further study (approximately 10% of total animals). Thirty minutes later, baseline was obtained by placing mice on an accelerating rotarod (4–40 rpm over 300 s). The latency to fall from the rotarod was recorded. Mice were then treated with haloperidol (1.0 mg/kg i.p.) or saline and retested 1, 2, and 4 h later for the duration to stay on the accelerating rotarod (4–40 rpm over 300 s).

**Western Blotting Analysis.** Spinal and supraspinal CaMKII $\alpha$  expression and activity were determined in naive and drug-treated mice using the Western blotting method, as we have described previously (Tang et al., 2006a; Luo et al., 2008; Chen et al., 2010; He et al., 2010). For consistency, tissues from frontal cortex and lumbar spinal sections were used to represent supraspinal and spinal samples. Tissues were homogenized in ice-cold radioimmunoprecipitation assay buffer, and solubilized samples (60- $\mu$ g protein) were separated by 12% SDS-polyacrylamide gel electrophoresis. After electrotransfer onto polyvinylidene difluoride membrane, the membrane was probed with a rabbit anti-Thr286-pCaMKII $\alpha$  antibody (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a mouse anti-CaMKII $\alpha$  antibody (1:1000; Santa Cruz Biotechnology, Inc.) at room temperature for 3 h, followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit (for pCaMKII $\alpha$ ) or anti-mouse (for CaMKII $\alpha$ ) secondary antibody (1:1000; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The specificities of these antibodies have been characterized in transgenic mice with CaMKII $\alpha$ T286A point mutation and in mice that were treated with small interfering RNA for CaMKII $\alpha$  (Chen et al., 2010). An enhanced chemiluminescence detection system (ECL; Thermo Fisher Scientific, Waltham, MA) was applied for detection. The membrane was then stripped and reprobed with a mouse anti- $\beta$ -actin antibody (1:10,000; Sigma-Aldrich) followed by a horseradish peroxidase-conjugated anti-mouse secondary antibody (1:1000; GE Healthcare) and developed as above. ECL signals were detected by a ChemiDoc system and analyzed using the Quantity One program (Bio-Rad, Hercules, CA). Ratios of the optical densities of CaMKII $\alpha$  or pCaMKII $\alpha$  to those of  $\beta$ -actin were calculated for each sample.

**Statistical Analysis.** All data are presented as mean  $\pm$  S.E.M. ED<sub>50</sub> values were obtained from dose-response curves using the method of Tallarida and Murray (1987). Comparisons between groups were analyzed using Student's *t* test (two groups) or a two-way repeated measure analysis of variance followed by post hoc analyses using Dunnett's *t* test (multiple groups). Statistical significance was established at 95% confidence limit.

## Results

**Prevention of Acute Opioid Tolerance and Dependence by Haloperidol.** We first investigated whether haloperidol could prevent the development of opioid tolerance and dependence. In the first series of experiments, an acute model of opioid tolerance and dependence was used. Mice received an induction dose of morphine (100 mg/kg s.c.) and were found 4.5 h later to exhibit significantly reduced antinociception ( $25.5 \pm 4.0\%$  MPE versus  $93.6 \pm 6.4\%$  MPE in saline-pretreated mice,  $p < 0.001$ ) by a test dose of morphine (10 mg/kg s.c.), indicative of the development of acute opioid tolerance (Fig. 1A). In mice pretreated with haloperidol (1 or 0.6 mg/kg i.p.) 30 min before the induction dose of morphine, morphine-antinociception remained largely intact ( $90.2 \pm 14.1$  and  $81.6 \pm 27.0\%$ , respectively; not significantly differ-



**Fig. 1.** Prevention of acute opioid tolerance (A) and dependence (B) by haloperidol. Separate groups of six mice received haloperidol (0.2, 0.6, and 1.0 mg/kg i.p.) or equal volume of saline 30 min before administration of morphine (100 mg/kg s.c.). Saline mice only received intraperitoneal injections of saline. Four hours later, all groups received a test dose of morphine (10 mg/kg s.c.). The antinociception was determined by the tail-flick assay 30 min later. A, development of morphine tolerance was prevented by haloperidol in a dose-dependent manner. B, development of morphine dependence, as revealed by naloxone (10 mg/kg i.p.)-precipitated withdrawal jumping, was also prevented by haloperidol in a dose-dependent manner. Data are expressed in mean  $\pm$  S.E.M. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  compared with the saline group; #,  $p < 0.05$ ; ###,  $p < 0.001$  compared with the morphine (MS) group.

ent from the saline group). Even at the lowest dose used, haloperidol (0.2 mg/kg i.p.) was able to partially prevent morphine-antinociceptive tolerance ( $59.0 \pm 38.7\%$  MPE,  $p < 0.05$  versus morphine alone) (Fig. 1A). These data demonstrated that haloperidol dose-dependently blocked the development of acute morphine tolerance.  $ED_{50}$  was estimated to be  $0.2 \pm 0.1$  mg/kg.

The acute model can also be used to study opioid dependence, as revealed by challenging morphine-treated mice with naloxone (10 mg/kg i.p.). These mice produced  $99 \pm 14$  jumps. Pretreatment with haloperidol (0.2, 0.6, and 1.0 mg/kg i.p.) reduced the numbers of naloxone-precipitated withdrawal jumps to  $72 \pm 16$ ,  $48 \pm 8$  ( $p < 0.001$ ), and  $43 \pm 7$  ( $p < 0.001$ ), respectively. Therefore, haloperidol prevented morphine physical dependence in a dose-dependent manner.

**Effects of Haloperidol on Basal Nociception, Morphine Antinociception, and Locomotor Activity.** To investigate whether haloperidol itself produced antinociception

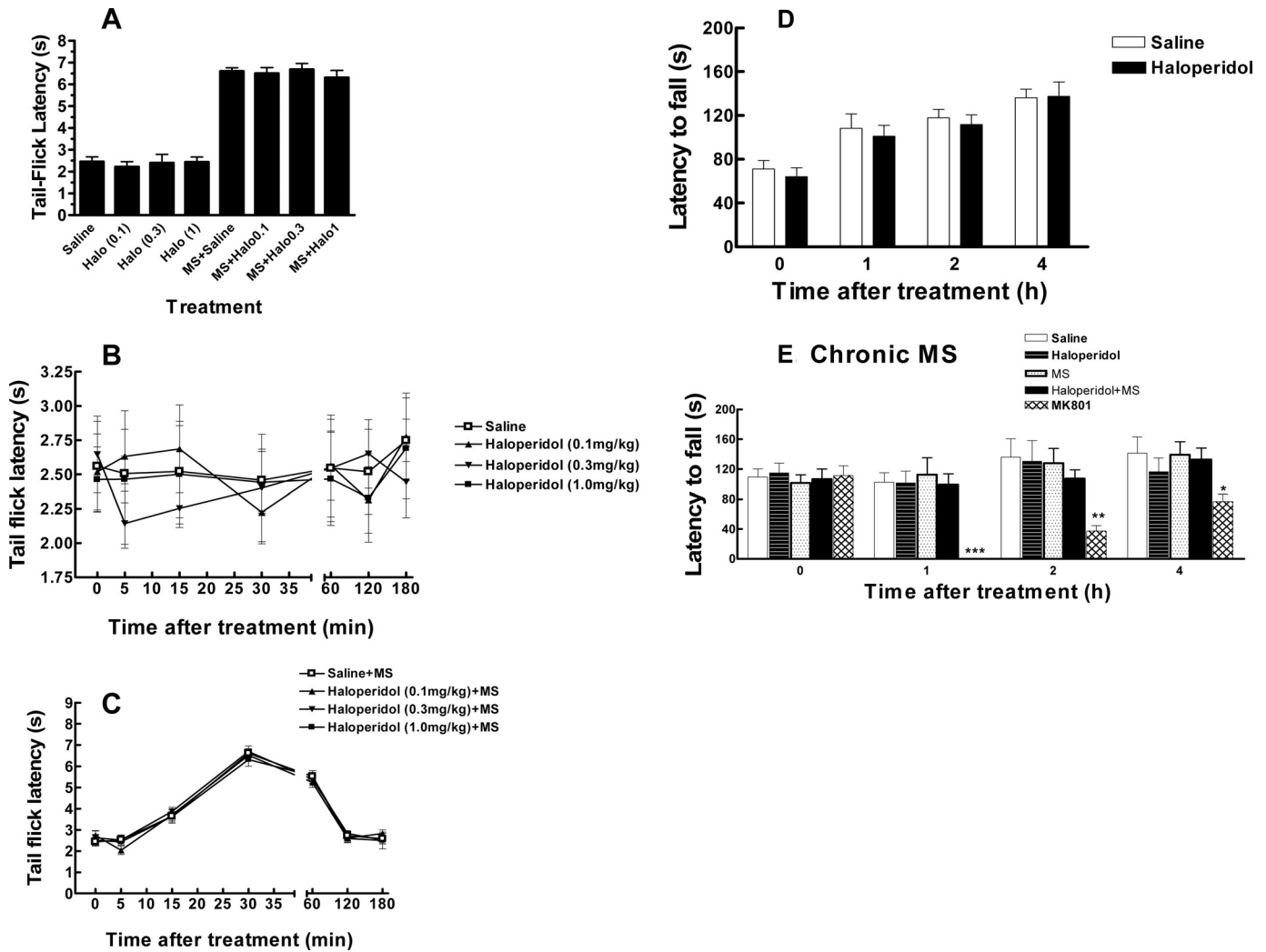
in naive mice or affected the antinociceptive effect of morphine, haloperidol (1.0 mg/kg i.p.) was either given alone or coadministered with a submaximal dose of morphine (3.0 mg/kg s.c.). The latter was used to avoid the ceiling effect so further enhancement of morphine antinociception could be detected. Haloperidol (0.1–1 mg/kg i.p.) by itself neither produced any antinociception effect compared with the saline group (Fig. 2A) nor altered morphine antinociception (Fig. 2A). The observation was further supported by monitoring the effect of these drugs for 3 h (Fig. 2, B and C).

Another potential confounding factor is that haloperidol may impair locomotor activity in animals. To address this possibility, we tested the effect of haloperidol on locomotor activity using a rotarod test (Chen et al., 2010). After two training sessions (24 h apart), mice were tested for their ability to stay on an accelerating rotarod at 0, 1, 2, and 4 h after the intraperitoneal administration of haloperidol (1.0 mg/kg) or saline. Haloperidol (1.0 mg/kg i.p.) did not affect the locomotor activity ( $p > 0.05$ , compared with the saline group) (Fig. 2D). These data suggested that the effect of haloperidol at doses of up to 1 mg/kg on morphine tolerance and dependence was not attributed to direct antinociceptive activity, interfering with morphine antinociception or impairment of locomotor activity. Haloperidol at a higher dose (3 mg/kg) seemed to cause sedation in ICR mice.

**Reversal of Acute Opioid Tolerance and Dependence by Haloperidol.** We next investigated whether short-term treatment with haloperidol was able to reverse the already established morphine-antinociceptive tolerance and physical dependence in the same acute mouse model. Haloperidol (0.60 mg/kg i.p.) completely reversed morphine tolerance ( $87.7 \pm 10.7\%$  MPE versus  $30.7 \pm 3.6\%$  by the morphine group,  $p < 0.001$ ), whereas at a lower dose (0.20 mg/kg i.p.), it showed a partial effect ( $64.8 \pm 11.7\%$  MPE). At the lowest dose used (0.06 mg/kg i.p.), it was ineffective ( $26.1 \pm 7.2\%$  MPE) (Fig. 3A).

Five hours after the induction dose of morphine, naloxone (10 mg/kg i.p.)-precipitated withdrawal jumping was evaluated. Haloperidol (0.06–0.60 mg/kg i.p.) dose-dependently attenuated the number of withdrawal jumps ( $p < 0.001$  compared with the morphine group; Fig. 3B). At the highest dose (0.60 mg/kg), haloperidol was able to completely suppress the withdrawal jumping. The drug at lower doses (0.06 and 0.20 mg/kg) also significantly reduced the number of withdrawal jumps (Fig. 3B).

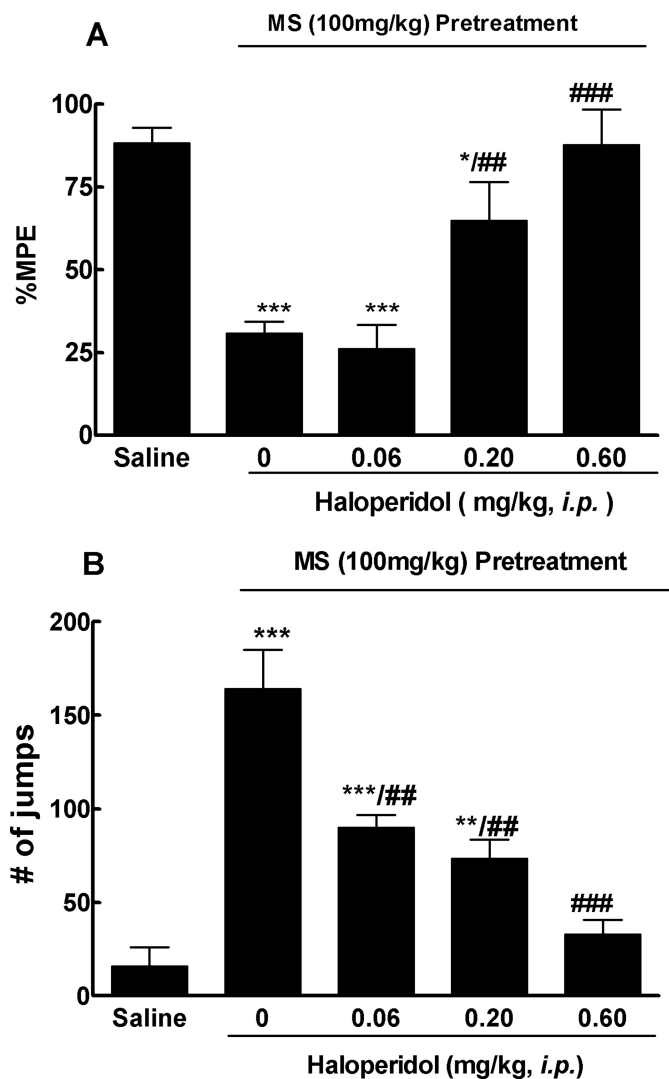
**Effect of Haloperidol on Brain and Spinal CaMKII $\alpha$  Activity in Acute Opioid Tolerance and Dependence.** To identify the potential cellular mechanism of haloperidol in opioid tolerance and dependence, we examined CaMKII $\alpha$  expression and activity in mice treated with morphine and different doses of haloperidol. Treatment with haloperidol for 0.5 to 5 h did not change the expression of CaMKII $\alpha$ . However, spinal CaMKII $\alpha$  activity was inhibited 30 min after the treatment with haloperidol. After morphine exposure, CaMKII $\alpha$  activity was significantly increased in the brain and spinal cord of morphine-treated mice (Fig. 4). Pretreatment with haloperidol (0.6 mg/kg i.p.) effectively prevented morphine-induced CaMKII $\alpha$  activation in the brain (Fig. 4A) and spinal cord (Fig. 4B) (lane 6, both  $p < 0.05$  compared with morphine group), correlating with its effect in preventing the development of tolerance to and dependence on morphine. Acute treatment with haloperidol (0.6 mg/kg i.p.) significantly reversed mor-



**Fig. 2.** Effects of haloperidol on basal antinociception and morphine antinociception (A–C) and locomotor activity in naive (D) and morphine-tolerant (E) mice. A, separate groups of six mice received haloperidol (Halo; 0.1, 0.3, or 1 mg/kg i.p.) or an equal volume of saline 30 min before the tail-flick test (left columns). Additional groups of six mice were pretreated with saline or haloperidol (0.1, 0.3, or 1 mg/kg i.p.) 30 min before a test dose of morphine (MS; 3 mg/kg s.c.) for antinociception test. Haloperidol at these doses (0.1–1 mg/kg) did not by itself produce antinociception or alter morphine antinociception. B, time course for the effect of haloperidol (0.1, 0.3, or 1 mg/kg i.p.) and saline in the tail-flick test. C, time course for the effect of haloperidol (0.1, 0.3, or 1 mg/kg i.p.) on antinociception produced by morphine (3 mg/kg s.c.). A to C,  $n = 6$ /group. D, effect of haloperidol on locomotor activity in naive mice. For the rotarod test, mice were trained to remain on a fixed speed (4 rpm) rotarod for 60 s. On the next day, mice were retrained, and baseline was obtained by placing mice on an accelerating rotarod (4–40 rpm over 300 s). Mice were then treated with haloperidol (1.0 mg/kg i.p.) or saline and retested 1, 2, and 4 h later. The latency to fall off of the rotarod was recorded.  $n = 6$ /group. No significant difference was identified. E, effect of haloperidol on locomotor activity in morphine-tolerant mice. Mice received a twice daily injection of morphine (10 mg/kg) to induce tolerance. Mice were trained on day 4, and the rotarod test was tested on day 5 as described above. Mice were treated with saline, haloperidol (1 mg/kg i.p.), haloperidol (1 mg/kg i.p.), and morphine (10 mg/kg s.c.), or MK801 (1 mg/kg i.p.). The latter is an NMDA receptor antagonist that is known to impair locomotor activity and was used as a positive control.  $n = 6$  for each test group and 12 for the saline group. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  compared with the saline group.

phine-induced CaMKII $\alpha$  activation in the brain and spinal cord. Haloperidol at a lower dose (0.2 mg/kg) attenuated supraspinal, but not spinal, activation of CaMKII $\alpha$  by morphine. At the lowest dose (0.06 mg/kg), haloperidol did not affect CaMKII $\alpha$  activity. No statistical difference was found for CaMKII $\alpha$  expression between control (saline) and treated (morphine or morphine plus haloperidol) mice in the brain or spinal cord (Fig. 4). Therefore, short-term haloperidol treatment dose-dependently reversed morphine-induced CaMKII $\alpha$  activation, which was in agreement with dose-dependent reversal of morphine tolerance and dependence. Furthermore, the increased CaMKII $\alpha$  activity was largely attributed to increased activation but not expression of the kinase.

**Reversal of Chronic Opioid Tolerance and Dependence by Haloperidol.** Given the promising effect of haloperidol in acute morphine tolerance and dependence, we further investigated the drug in chronic models of morphine tolerance and dependence. Mice received morphine sulfate (10 mg/kg s.c.) or saline every 12 h. Morphine tolerance quickly developed and reached the peak around day 5 (Tang et al., 2006a; He et al., 2010) when the test dose of morphine (10 mg/kg) produced significantly reduced antinociception ( $14.1 \pm 3.8\%$  MPE), indicative of the presence of opioid tolerance (Fig. 5A). Short-term treatment with haloperidol (1.0 mg/kg i.p.) completely reversed morphine tolerance; the test dose of morphine remained highly active and pro-



**Fig. 3.** Reversal of acute opioid tolerance (A) and dependence (B) by haloperidol. Separate groups of six mice received morphine (100 mg/kg s.c.) or an equal volume of saline. Four hours later, haloperidol (0.06, 0.20, and 0.60 mg/kg i.p.) or saline was given to these mice. Thirty minutes later, all groups received a test dose of morphine (10 mg/kg s.c.) for the antinociception test. A, the established morphine-antinociceptive tolerance was reversed by haloperidol in a dose-dependent manner. B, development of morphine dependence, as revealed by 10 mg/kg i.p. naloxone-precipitated withdrawal jumping, which was also reversed by haloperidol in a dose-dependent way. Data are expressed as mean  $\pm$  S.E.M. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  compared with the saline group; ##,  $p < 0.01$ ; ###,  $p < 0.001$  compared with the morphine (MS) alone group.

duced significant antinociception ( $95.5 \pm 5.3\%$  MEP,  $p < 0.001$ ; Fig. 5A). The drug at a lower dose (0.3 mg/kg i.p.) partially attenuated morphine-antinociceptive tolerance ( $31.7 \pm 9.7\%$  MPE), whereas it was ineffective ( $12.3 \pm 4.5\%$  MPE) at the lowest dose (0.1 mg/kg i.p.). Likewise, haloperidol at the highest dose (1.0 mg/kg i.p.) completely blocked naloxone-precipitated withdrawal jumping (Fig. 5B), although it did have significant effect on the lower doses (0.3 and 0.1 mg/kg i.p.).

Although haloperidol (1.0 mg/kg i.p.) was found not to impair locomotor activity in naive mice, it was not known whether the drug had a different effect on opioid-tolerant mice. Therefore, we further determined the effect of haloperidol in the rotarod test in morphine-tolerant mice (10 mg/kg,

twice daily for 5 days). After two training sessions (on days 4 and 5), mice were tested for their ability to stay on an accelerating rotarod at 0, 1, 2, and 4 h after the intraperitoneal administration of saline, haloperidol (1.0 mg/kg), haloperidol (1.0 mg/kg), and morphine (10 mg/kg s.c.) or MK801 [1.0 mg/kg; (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine maleate]. MK801, an NMDA receptor antagonist that is known to impair rotarod performance, was used as a positive control. Unlike MK801, haloperidol (1.0 mg/kg) did not affect the locomotor activity (Fig. 2E). These data suggested that the effect of haloperidol on chronic morphine tolerance and dependence was not the result of impaired locomotor activity.

Because haloperidol is effective when taken orally in humans, we further examined whether haloperidol administered orally was effective in reversing chronic morphine tolerance and dependence. On day 5, haloperidol (0.1–1.0 mg/kg, gastric gavage) was administered 30 min before the test dose of morphine (10 mg/kg s.c.). As expected, haloperidol administered orally dose-dependently attenuated both morphine-antinociceptive tolerance (Fig. 5A) and morphine physical dependence (Fig. 5B).

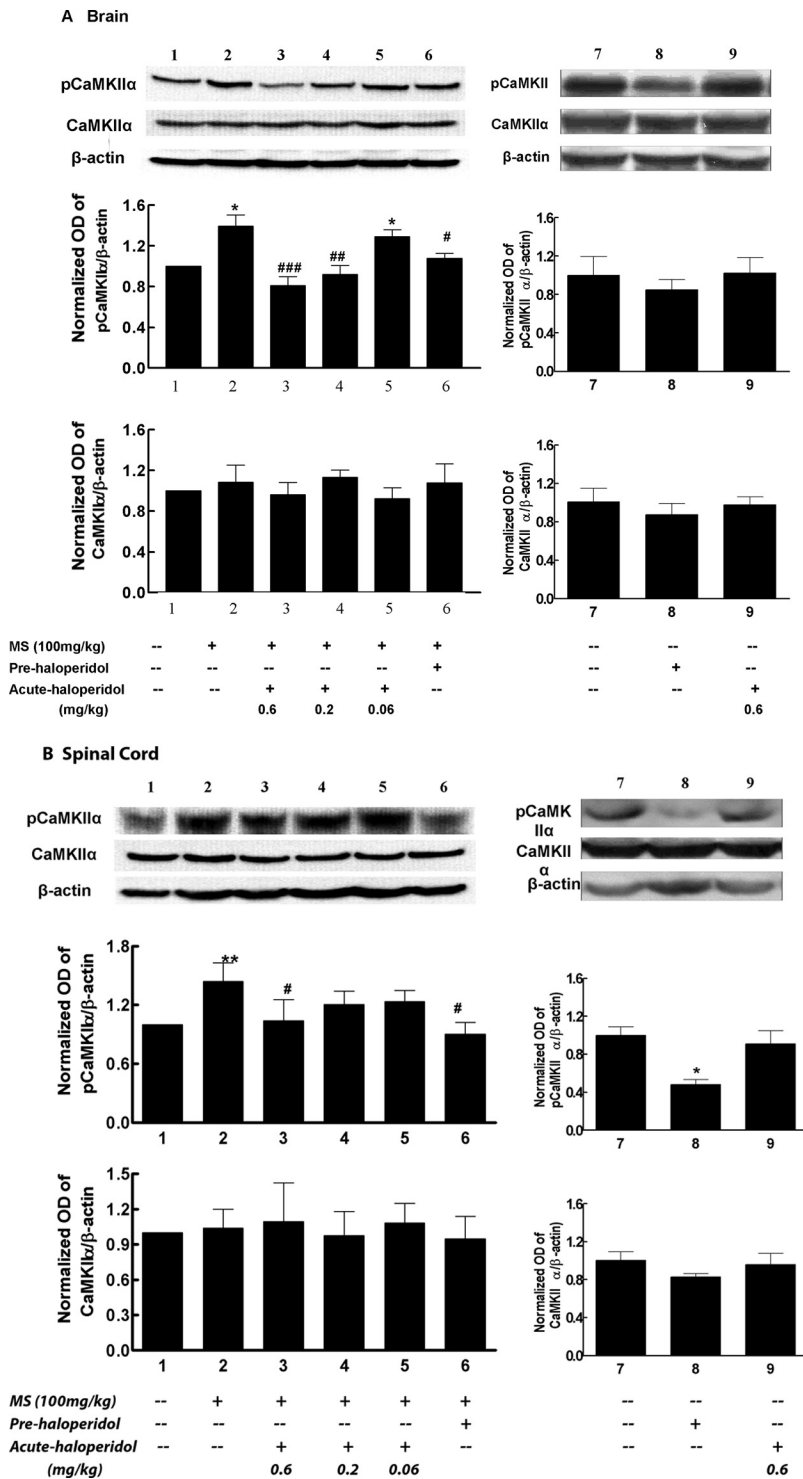
#### Effect of Haloperidol on CaMKII $\alpha$ Activity in Mice Chronically Tolerant to and Dependent on Morphine.

To correlate behavioral effect with biochemical inhibition of CaMKII $\alpha$  by haloperidol, brain (frontal cortex) samples were taken from mice that have been treated chronically with morphine (10 mg/kg s.c., twice daily, for 5 days) and acutely with haloperidol (approximately 0.1–1.0 mg/kg i.p. or p.o.). Haloperidol (1.0 mg/kg) either given intraperitoneally (Fig. 6A) or orally (Fig. 6B) completely blocked morphine-induced activation of CaMKII $\alpha$  (lane 3). At the second highest dose (0.3 mg/kg, lane 4), haloperidol partially blocked CaMKII $\alpha$  activation after intraperitoneal administration (Fig. 6A,  $p < 0.01$  compared with the morphine group), but the effect was not significant when given orally (Fig. 6B). At 0.1 mg/kg, haloperidol did not alter CaMKII $\alpha$  activation by morphine by either route of administration.

## Discussion

In this study, we demonstrated that haloperidol disrupted opioid-antinociceptive tolerance and physical dependence in two rodent models. The behavioral effect of haloperidol seemed to correlate with its inhibitory effect on CaMKII $\alpha$  in opioid-tolerant and -dependent state. These data are in agreement with our previous studies that have suggested a critical role of CaMKII $\alpha$  and its signaling pathways in opioid tolerance and dependence (Wang et al., 2003; Tang et al., 2006a).

It has been demonstrated that CaMKII $\alpha$  can phosphorylate the NMDA receptor (McGlade-McCulloh et al., 1993; Lau and Huganir, 1995), leading to enhanced NMDA receptor activity and influx of Ca<sup>2+</sup> through the channels (Kitamura et al., 1993). The latter, in turn, results in more activation of calmodulin (Klee et al., 1980; Shifman et al., 2006) and autophosphorylation of CaMKII $\alpha$  at position Thr286 (Fukunaga et al., 1992; Strack and Colbran, 1998). This positive feedback between CaMKII $\alpha$  and NMDA receptor can serve as one mechanism for sustained activation of CaMKII $\alpha$  and NMDA receptors in opioid tolerance and dependence. NMDA receptor activation is a key mechanism promoting opioid



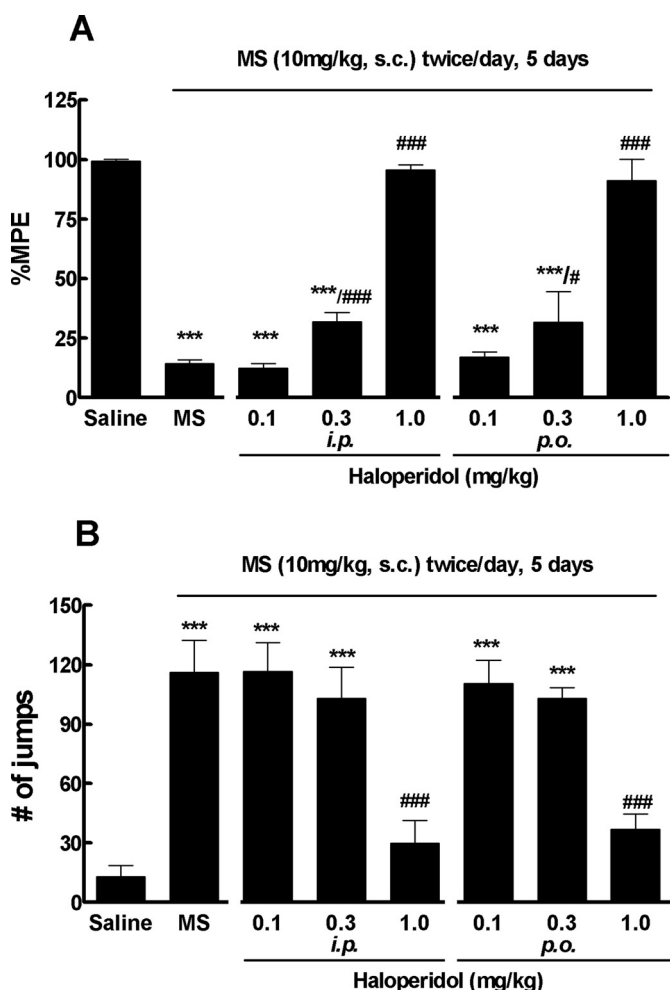
**Fig. 4.** Effect of haloperidol on supraspinal (A) and spinal (B) CaMKII $\alpha$  activity in opioid-tolerant and -dependent mice. Frontal cortex (A) and spinal lumbar section (B) samples were taken to determine CaMKII $\alpha$  activity. For acute haloperidol effect, mice were pretreated with morphine (100 mg/kg) or saline at time 0, followed by haloperidol (0.6 mg/kg) or saline at time = 4.5 h, with samples taken at time = 5 h. For the pretreatment effect, mice received morphine (100 mg/kg) and haloperidol (0.06, 0.2, 0.6 mg/kg) or saline at time 0, and samples were taken at time = 5 h. The activated CaMKII $\alpha$  and total CaMKII $\alpha$  were determined by the Western blotting method using antibodies specific for Thr286-pCaMKII $\alpha$  and CaMKII $\alpha$ , respectively. Histogram data, expressed as mean  $\pm$  S.E.M., were constructed from the representative figure shown and three other experiments ( $n = 4$ ). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  compared with the saline group; #,  $p < 0.05$ ; ##,  $p < 0.01$ ; ###,  $p < 0.001$  compared with the morphine (MS) alone group.

tolerance and dependence (Trujillo and Akil, 1991; Noda and Nabeshima, 2004).

As a major serine/threonine protein kinase, CaMKII $\alpha$  is expected to have many downstream effectors in addition to the NMDA receptor. Of these, the  $\mu$ -opioid receptor ( $\mu$ OR) is arguably the most relevant for opioid tolerance and dependence. CaMKII has been reported to modulate desensitization of  $\mu$ OR in cells (Mestek et al., 1995; Koch et al., 1997). This was further supported by the findings that  $\mu$ OR and CaMKII $\alpha$  are colocalized in dorsal root ganglion neurons and in the superficial

laminae of the spinal cord dorsal horn (Brüggemann et al., 2000). CaMKII $\alpha$  has also been reported to phosphorylate glycosylated phosphocin-like protein after long-term treatment with morphine (Sánchez-Blázquez et al., 2008).

The typical antipsychotic drug haloperidol has been used clinically for decades. Its antipsychotic effect is thought to be mediated by blocking the dopamine D2 receptor (Leucht et al., 2008). Haloperidol is approximately 40 times more selective to the D2 receptor than it is to the D1 receptor (Leucht et al., 2008). A high level of D2 receptor occupancy was found

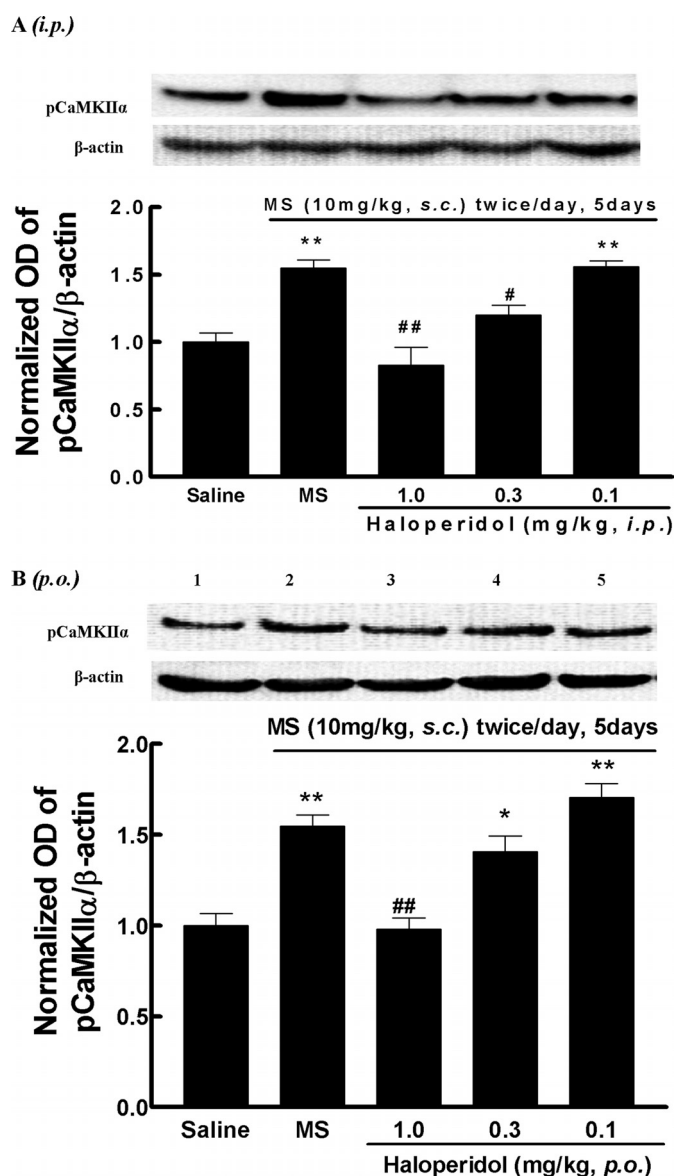


**Fig. 5.** Reversal of chronic morphine tolerance and dependence by haloperidol in mice. Separate groups of six mice received morphine (10 mg/kg s.c.) or an equal volume of saline twice daily for 5 days. Haloperidol dose-dependently reversed the established morphine-antinociceptive tolerance (A) and naloxone (10 mg/kg i.p.)-precipitated withdrawal jumping (B). Data are expressed in mean  $\pm$  S.E.M. \*\*\*,  $p < 0.001$  compared with the saline group; #,  $p < 0.05$ ; ###,  $p < 0.001$  compared with the morphine (MS) alone group.

after a relatively low dose of haloperidol in humans (Kapur et al., 1996).

The interaction between the dopamine and opiate systems is well recognized (Unterwald and Cuntapay, 2000). Considerable evidence suggests that dopamine activity affects the opioid system by modulating opiate peptide transcripts (Morris and Hunt, 1991), synthesis (Voorn et al., 1994), release, and biotransformation (Hong et al., 1985; Terashvili et al., 2008) (Li et al., 1986). In contrast, opioids modulate the dopamine system by several mechanisms, such as dopamine synthesis (Alper et al., 1980), release (Di Chiara and Imperato, 1988; Devine et al., 1993), biotransformation (Yonehara and Clouet, 1984), and activity of dopaminergic neurons (Walker et al., 1987; Johnson and North, 1992).

In addition, haloperidol has been reported to potentiate antinociception of morphine in the rat, possibly by acting as a  $\sigma$ -receptor antagonist (Chien and Pasternak, 1995). This would raise the possibility that haloperidol could have disrupted opioid tolerance by merely potentiating opioid analgesia. However, we found that haloperidol inhibited CaMKII activity in morphine-treated mice. Previous studies using



**Fig. 6.** Effect of haloperidol on brain CaMKII $\alpha$  activity in opioid-tolerant and -dependent mice (chronic model). Separate groups of three mice received morphine (10 mg/kg s.c.) or an equal volume of saline twice per day for 5 days. Thirty minutes before the last injection of morphine on day 5, mice were treated with haloperidol (A) (approximately 0.1–1.0 mg/kg i.p.), haloperidol (approximately 0.1–1.0 mg/kg p.o.) (B), or saline. Brain samples were taken 30 min after the last injection of morphine or saline. The activated CaMKII $\alpha$  and total CaMKII $\alpha$  were determined by the Western blotting method using antibodies specific for Thr286-pCaMKII $\alpha$  and CaMKII $\alpha$ , respectively. Histogram data, expressed as mean  $\pm$  S.E.M., were constructed from the representative figure shown and three other experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  compared with the saline group; #,  $p < 0.05$ ; ##,  $p < 0.01$  compared with the morphine (MS) alone group.

chemical inhibitors and genetic manipulation, which are not expected to affect  $\sigma$ -receptor activity, demonstrated that inhibition of CaMKII led to diminished opioid tolerance and dependence (Fan et al., 1999; Tang et al., 2006a). Moreover, we found that haloperidol (up to 1 mg/kg) did not interfere with morphine antinociception, which may indicate that such an action is dose-dependent and may also differ among animal species (or strain). We found obvious signs of sedation in mice that were treated with haloperidol at 3 mg/kg; therefore, we did not pursue further studies using doses higher

than 1 mg/kg. Haloperidol (up to 1 mg/kg) did not by itself produce antinociception effect, which was in agreement with a previous report (Chien and Pasternak, 1995).

On the other hand, haloperidol has also been shown to antagonize the effects of opioids in other experimental settings (Cowan et al., 1986; Cheido and Idova, 2007). Whereas haloperidol increased the biosynthesis and release of endogenous opioid peptides from the myenteric plexus (Milanés et al., 1985), the drug is also known to down-regulate  $\mu$ OR in certain brain regions (Mavridis and Besson, 1999; Bower et al., 2000). Some of these discrepancies again could be attributed to the different doses used. However, these results were largely interpreted by its direct effect of blocking the dopamine D2 receptor. In fact, some of the pharmacological effects of haloperidol may be attributed to its actions at CaMKII. For example, it has been previously demonstrated that morphine-conditioned place preference was reversed by the treatment of a high dose of haloperidol (Manzanedo et al., 2001). The effect may also be explained by its inhibition of CaMKII. Furthermore, CaMKII can positively regulate the D2 receptor signaling (Greenstein et al., 2007) and expression (Takeuchi et al., 2002).

The D2 dopamine agonists enhanced the ability of MK801 to attenuate the development of morphine tolerance and dependence (Verma and Kulkarni, 1995). However, the physical signs of opioid withdrawal were not altered in mice lacking the D2 dopamine receptor (Maldonado et al., 1997), indicating that attenuation of opioid dependence by haloperidol was not attributed to blocking the D2 receptor.

In summary, we found that haloperidol prevented and reversed morphine-induced CaMKII $\alpha$  activation, antinociceptive tolerance, and physical dependence. These data not only provide additional support for the role of CaMKII $\alpha$  in opioid tolerance and dependence but also is a step forward in the direction of translational application, especially because the drug was effective when given orally. These data raise the possibility of applying haloperidol to prevent or treat opioid dependence and to improve pain treatment by attenuating opioid tolerance and opioid-induced hyperalgesia.

#### Authorship Contributions

*Participated in research design:* Yang, Chen, Tang, and Wang.  
*Conducted experiments:* Yang, Chen, and Tang.  
*Performed data analysis:* Yang, Chen, and Wang.  
*Wrote or contributed to the writing of the manuscript:* Yang, Chen, and Wang.  
*Other:* Wang acquired funding for the research.

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**Address correspondence to:** Dr. Zaijie Jim Wang, Department of Biopharmaceutical Sciences (MC 865), University of Illinois, 833 South Woods Street, Chicago, IL 60612. E-mail: zjwang@uic.edu

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