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Reversal of morphine antinociceptive tolerance and dependence by the acute supraspinal
inhibition of Ca^{2+} /calmodulin-dependent protein kinase II

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List of abbreviations

CaMKII: Ca²⁺/calmodulin-dependent protein kinase II

CREB: cAMP response element-binding protein

GIRK: G-protein gated inwardly rectifying potassium channels

NMDA: N-methyl-D-aspartate

μOR: μ opioid receptor

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LTP: long-term potentiation

MPE: maximal possible effect

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ABSTRACT

Previous studies have suggested that Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) can modulate opioid tolerance and dependence via its action on learning and memory. In this study, we examined if CaMKII could directly regulate opioid tolerance and dependence. CaMKII activity was increased after the treatment with morphine (100mg/kg, *s.c.*, or 75mg morphine/pellet/mouse, *s.c.*); the effect exhibited a temporal correction with the development of opioid tolerance and dependence. In mice treated with morphine (100mg/kg, *s.c.*), morphine tolerance and dependence developed in 2-6 h. An acute supraspinal administration of KN93, a CaMKII inhibitor, was able to dose-dependently reverse the already-established antinociceptive tolerance to morphine ($p < 0.001$ for 15-30 nmol; not significant for 5 nmol). KN92 (30 nmol, *i.c.v.*), a kinase-inactive analogue of KN93, did not affect opioid tolerance. Neither KN92 nor KN93 affected basal nociception or acute morphine (1-10 nmol, *i.c.v.*)-antinociception. Similarly, dependence on morphine was abolished by the acute administration of KN93, but not KN92, in a dose-dependent manner. Pretreatment of mice with KN93 also prevented the development of morphine tolerance and dependence. The effect of acute CaMKII inhibition was not limited to the particular experimental model, as KN93 also acutely reversed the established opioid tolerance and dependence in mice treated with morphine (75mg/ pellet/mouse, *s.c.*) for 6 days. Taken together, these data strongly support the hypothesis that CaMKII can act as a key and direct factor in promoting opioid tolerance and dependence. Identifying such a direct mechanism may be useful for designing pharmacological treatments for these conditions.

Introduction

Opioids, such as morphine, are clinically used primarily as analgesics. Drug tolerance and dependence are two of the major problems associated with these drugs, which greatly limit their effectiveness and usage. The molecular mechanisms underlying opioid tolerance and dependence are not entirely understood. The current study aims to test the hypothesis that calcium (Ca^{2+})/calmodulin-dependent protein kinase II (CaMKII) can directly regulate opioid tolerance and dependence.

CaMKII is a multifunctional, Ca^{2+} /calmodulin-activated protein kinase, whose α and β isoforms are abundant in the central nervous system (Hudmon and Schulman, 2002). A vast amount of information is available for the interaction of CaMKII α isoform and N-methyl-D-aspartate (NMDA) receptors in generating long-term potentiation (LTP) in hippocampal neurons, which is critical for learning and memory (e.g., Mayford et al., 1996). Inhibition or disruption of CaMKII impairs spatial learning (Silva et al., 1992). Interestingly, antagonists of the NMDA receptor effectively inhibit the development of opioid tolerance and dependence (Marek et al., 1991; Trujillo and Akil, 1991). It has also been reported that chronic microinjection of CaMKII inhibitors into hippocampus was able to prevent the development of opioid tolerance (Fan et al., 1999). The same CaMKII inhibitors were ineffective when chronically microinjected into striatum. Further, acute administration of these CaMKII inhibitors into hippocampus did not affect opioid tolerance. These data supported the importance of learning and memory pathways in opioid tolerance (Fan et al., 1999).

These studies, however, did not address the possibility that CaMKII could directly (i.e., not necessarily relying on learning and memory) modulate opioid tolerance and dependence. Identifying such a direct mechanism for CaMKII is not only important to our

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understanding, but will also have a profound impact on designing therapeutic interventions for opioid tolerance and dependence. Studies have suggested that the NMDA system may be directly involved in promoting opioid tolerance and dependence (Gutstein and Trujillo, 1993). Similar direct mechanisms have not been proposed for CaMKII.

In cellular models of opioid tolerance, μ opioid receptor (μ OR) desensitization was enhanced when a constitutively active form of CaMKII was also expressed in *Xenopus* oocytes (Mestek et al., 1995; Koch et al., 1997). The effect was absent if the native receptor was replaced with a mutated receptor lacking the consensus CaMKII phosphorylation sites (Koch et al., 1997). On the other hand, intracellular Ca^{2+} , calmodulin, and CaMKII can all be regulated by opioids. Cytosolic free Ca^{2+} was increased after the treatment with opioids (Fields and Sarne, 1997; Smart et al., 1997; Spencer et al., 1997; Quillan et al., 2002). Similarly, chronic treatments with opioids have been found to increase calmodulin activity (Nehmad et al., 1982) and mRNA levels (Niu et al., 2000). Indeed, CaMKII activity was increased in opioid tolerance *in vivo* (Lou et al., 1999; Wang et al., 2003; Liang et al., 2004). Anatomically, CaMKII and μ OR were found to be co-localized in dorsal root ganglia sensory neurons and superficial layers of spinal cord dorsal horn (Bruggemann et al., 2000). Moreover, upon activation, μ OR was found to internalize to intracellular locations where CaMKII proteins were located (Bruggemann et al., 2000). Therefore, cellular and biochemical evidences support the possibility that CaMKII and opioid system can directly interact with each other, leading to cellular opioid desensitization. In a preliminary study, we found that tolerance to morphine was abolished by an acute spinal treatment with a single dose of KN93, a CaMKII inhibitor (Niki et al., 1993), consistent with the hypothesis that CaMKII can directly modulate opioid tolerance (Wang et al., 2003). However, we caution that multiple factors can confound this interpretation. The current study extends our previous

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findings, by carefully applying different degree of CaMKII inhibition using multiple doses of KN93 as well as a negative control KN92 (Tombes et al., 1995) in two rodent models of opioid tolerance. In addition, we test in this study if opioid dependence can be directly regulated by CaMKII.

Materials and Methods

Materials

Morphine and placebo pellets were obtained from the National Institute on Drug Abuse (Rockville, MD). Morphine sulfate was from Abbott Laboratories (North Chicago, IL). 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN93) and 2-[N-(4-Methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN92) were purchased from Calbiochem (San Diego, CA). Naloxone and all other chemicals were of analytical grade or better from Sigma (St. Louis, MO). ICR mice (20-25 g, Harlan Laboratories, Indianapolis, IN) were maintained on a 12/12 h light/dark cycle and provided food and water *ad libitum* before experimental procedures. All experiments were performed in accordance with the NIH guidelines and after approval by the Animal Care and Use Committee of the University of Illinois at Chicago.

Drug administration

Introcerebroventricular (*i.c.v.*) injections, under light ether anesthesia, were made into the left lateral ventricles as described previously (Bilsky et al., 1996; Wang et al., 2001). Standard procedures were used for *i.p.* and *s.c.* injections. Placebo and morphine pellets were implanted in the *s.c.* space as described previously (Way et al., 1969; Patrick et al., 1975).

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Tests for antinociception

Basal nociception and morphine-induced antinociception were evaluated using the tail immersion test as described previously (Wang et al., 2001). Briefly, the test was performed by dipping the distal 1/3 of the tail into a water bath maintained at 52 °C, and recording the latency to a rapid tail flick response. Morphine (*i.c.v.*)-induced antinociception was tested at the time of peak drug response after the injection of morphine, and expressed as % of maximal possible effect (MPE) according to the following formula: %MPE=100*(postdrug latency-predrug latency)/(cut off- predrug latency). A 12 s cut-off was applied to prevent tissue injury. The time of peak drug response was determined to be 20 min in our studies, and was not altered by opioid tolerance (see Supplemental Data).

Acute opioid tolerance and dependence

Separate groups of 8 ICR mice (20-25 g) were made acutely tolerant to and dependent on opioids by the administration of a large dose of morphine (100mg/kg, *s.c.*) (Wang et al., 1994; Bilsky et al., 1996). We have reported that maximal morphine tolerance and dependence developed over 2-6 h (Bilsky et al., 1996). Control mice received the same volume of saline. Tolerance to opioids was studied in these mice 4.5 h later by measuring the antinociceptive effect exhibited by a test dose morphine (1-10 nmol, *i.c.v.*). A significant reduction of antinociceptive effect signified the presence of tolerance to morphine. To assess dependence, mice were given naloxone (1-10 mg/kg, *i.p.*) 5 hr after the administration of morphine (100mg/kg, *s.c.*), and immediately placed inside glass cylinders. Vertical jumps were recorded for 15 min. To determine the effect of CaMKII inhibition, separate groups of mice were given the CaMKII inhibitor KN93 (5-30 nmol, *i.c.v.*) (Niki et al., 1993) or its kinase-inactive structural analogue KN92 (30 nmol, *i.c.v.*) (Tombes et al., 1995) 15 min before

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naloxone or the test dose morphine (“reversal studies”). To test if KN93 or KN92 prevented the development of tolerance and dependence, KN93 or KN92 was co-administrated with morphine (100mg/kg, *s.c.*)

Effect of KN93 and KN92 on basal nociception and morphine-antinociception

To determine if CaMKII inhibitor KN93 or its inactive structural analogue KN92 affected tail-flick latency and interfered with morphine-antinociception, groups of 8 mice were given KN93 (30 nmol/5 μ l, *i.c.v.*), KN92 (30 nmol/5 μ l, *i.c.v.*) or saline (5 μ l, *i.c.v.*), 15 min before the administration of morphine (1-10 nmol, *i.c.v.*) or saline (5 μ l, *i.c.v.*). Latencies to tail-flick responses were determined 20 min later.

Chronic model of opioid tolerance and dependence

On Day 0, separate groups of 6 male ICR mice (20-25 g) were implanted subcutaneously with morphine pellets (1 pellet/mouse, each pellet contains 75 mg morphine base) to induce opioid tolerance and dependence. Control mice received placebo pellets (1 pellet/mouse, a placebo pellet is made of same excipients, but contains no morphine). Tolerance and dependence develop over the course of 2-6 days (Ho et al., 1975; Patrick et al., 1975). To determine morphine tolerance, mice were injected with a test dose morphine (10 nmol, *i.c.v.*). A significant reduction of morphine-antinociceptive effect signified the presence of tolerance to morphine. The presence of opioid dependence was revealed by challenging morphine-treated mice with naloxone (10 mg/kg, *i.p.*) on Day 6. Immediately after the administration of naloxone, mice were placed inside glass cylinders, and the number of withdrawal jumps were recorded for 15 min. To determine the effect of acute CaMKII inhibition on tolerance or dependence, separate groups of mice were given KN93 (5 - 45 nmol, *i.c.v.*) 15 min before

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naloxone or the test dose of morphine on Day 6.

Western blot analysis

Western blotting procedures were performed as previously described (Wang et al., 2001) .

Briefly, brain cortices were dissected and quickly frozen on dry ice. Tissues from 3 mice of the same experimental group were pooled and homogenized with a glass homogenizer in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA in PBS, pH 7.4) (3mL/g wet tissue) in the presence of protease inhibitors (0.05 mg/mL bestatin, 0.05mg/mL leupeptin, 0.05 mg/mL pepstatin, 0.1 mg/mL PMSF). The homogenates were incubated on a rotator at 4°C for 2 h, and the soluble fraction was collected after separation by centrifugation (45,000 x g, 60 min). Protein content was determined by a modified Bradford method (Pierce Biotechnology, Rockford, IL). Samples (15 µg protein) were separated by 10% SDS-PAGE and electro-transferred onto nitrocellulose membrane. The membrane was preblocked in 5% non-fat milk in 20 mM Tris-buffer saline (pH 7.6) with 0.1% Tween 20 and probed with rabbit antibodies recognizing CaMKII (1/1,000, Santa Cruz Biotechnology, Santa Cruz, CA), the activated form of CaMKII (anti-pCaMKII antibody, 1/1,000, Promega, Madison, WI), or the activated form of CREB (anti-pCREB antibody, 1/1,000, Santa Cruz). The membrane was then washed and incubated with an HRP-conjugated donkey anti-rabbit secondary antibody (1/1,000, Amersham, Piscataway, NJ), washed and developed using an enhanced chemiluminescence (ECL) detection system (Amersham). The membrane was then stripped and re-probed with the mouse anti-β-actin antibody (1/10,000, Sigma), followed by another incubation with anti-mouse HRP-conjugated secondary antibody (1/20,000, Amersham), and developed as above. ECL-signals were captured by a ChemiDoc imaging system and analyzed using Quantity One program (Biorad,

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Hercules, CA). Ratios of the optical densities of pCaMKII to that of β -actin were calculated for each sample.

Statistical analysis. Data are expressed in Mean \pm SEM (standard error). Differences in responses between the treatment groups were determined using ANOVA followed by Student's t (two groups) or Dunnett's t (multiple groups) tests. Statistical significance is established at 95%.

Results

Effect of the acute CaMKII inhibition on acute morphine tolerance

We first tested our hypothesis in an acute model of opioid tolerance. Tolerance to morphine was established in 2-6h after the administration of morphine (100mg/kg, *s.c.*) (Bilsky et al., 1996), as evidenced by the significant reduction of morphine (*i.c.v.*)-induced antinociception (Fig. 1A&B). Brain CaMKII activity increased over a time course of 1-4 h post *s.c.* morphine (Fig. 1C), correlated with the time course of the development of tolerance (Bilsky et al., 1996). KN93 (15-30 nmol, *i.c.v.*), a selective CaMKII inhibitor, administrated 35 min before the antinociceptive test (15 min before the *i.c.v.* test dose morphine), reversed the already-established tolerance to morphine. The effect was KN93 dose dependent, as KN93 at higher doses (15 and 30 nmol) was able to significantly reverse the established tolerance to morphine ($p < 0.001$), while ineffective at a lower dose (5 nmol) (Fig. 1A & B). In contrast, KN92, a kinase-inactive structural analog of KN93, had no effect on morphine antinociceptive tolerance (Fig. 1B). The western blotting experiments verified that acutely administrated KN93 (*i.c.v.*) significantly reduced the supraspinal CaMKII activity in morphine-treated mice (Fig. 1D).

Effect of KN93 and KN92 on basal nociception and morphine antinociception

One potential problem in interpreting the above data was that KN93 might directly affect basal nociception or interfere with the antinociceptive effect of morphine. To account for this possibility, we tested the effects of KN93 and KN92 on basal nociception and morphine-antinociception. KN93 or KN92 did not alter the basal tail-flick withdrawal latencies (data not shown). In all three morphine doses examined, morphine-induced antinociception was not affected by the administration of KN93 or KN92 (Fig. 2), indicating that KN93 or KN92 did not affect acute morphine antinociception.

Effect of the acute CaMKII inhibition on acute morphine dependence

In morphine (100mg/kg, s.c.)-treated mice, dependence on opioids developed in 2-6 h (Bilsky et al., 1996). Challenging these mice with naloxone (*i.p.*, 5 h post morphine) precipitated withdrawal jumps in a naloxone-dose dependent manner, which was largely absent in saline-treated control mice (Fig. 3A & B). KN93 (30 nmol, *i.c.v.*), given 15 min before naloxone, was able to completely suppress the naloxone-induced withdrawal jumping (Fig. 3 A & B). Withdrawal jumping was significantly attenuated ($p < 0.01$) at a lower dose (15 nmol), whereas KN93 at the lowest dose used (5 nmol) did not significantly affect naloxone-induced withdrawal jumping. Neither did the negative control compound KN92 (30 nmol) show a significant effect (Fig. 3B).

Prevention of opioid tolerance and dependence by the CaMKII inhibition

We next tested if a broad supraspinal inhibition of CaMKII could prevent the development of tolerance to and dependence on opioids. In these studies, KN93 or KN92 (30

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nmol, *i.c.v.*) was administered immediately before the injection of morphine (100 mg/kg, *s.c.*). Five hours later, mice received morphine or “morphine plus KN92” developed antinociceptive tolerance to morphine, whereas tolerance was absent in mice received “morphine plus KN93” ($p < 0.01$ compared with “MS” group; not significantly different from the control group) (Fig. 4A). Similar co-treatment with KN93, but not KN92 (not significantly different from the “MS” group), prevented the development of opioid dependence by significantly reducing the numbers of naloxone-induced withdrawal jumps ($p < 0.01$ compared with “MS” group; not significantly different from the control group) (Fig. 4B). These data indicated that a broad supraspinal inhibition of CaMKII was effective in preventing the development of both opioid tolerance and dependence in mice.

KN93 reversed opioid tolerance in a chronic model of opioid tolerance

To ascertain that the pharmacological effect of CaMKII inhibition was not limited to a particular animal model of opioid tolerance, we further tested our hypothesis in a chronic model of opioid tolerance. Mice developed tolerance to opioids over 2-6 days after receiving the *s.c.* implantation of morphine pellets (Ho et al., 1975; Patrick et al., 1975). The antinociception produced by morphine (10 nmol, *i.c.v.*) before the implantation of pellets was 91.0 ± 5.0 %MPE. On Day 6, the same test dose morphine produced a significantly reduced antinociceptive response in morphine-pelleted mice (11.5 ± 2.0 %MPE, $p < 0.001$), while remaining fully active in mice implanted with placebo pellets (93.1 ± 4.0 %MPE) (Fig. 5A), indicating the presence of antinociceptive tolerance in morphine-pelleted mice. Supraspinal CaMKII expression and activity increased significantly over the time course of 6 days as the tolerance developed (Fig. 5 B-D). When KN93 (15-45 nmol, *i.c.v.*) was given 15 min before morphine-antinociceptive tests, KN93 was able to significantly reverse the established

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tolerance in these mice ($p < 0.001$ for all three doses) (Fig. 5A). KN93 at the lowest dose used (5nmol) was not effective. Compared with the acute model, a higher dose of KN93 (45 nmol) was needed to achieve a complete reversal of tolerance (not significantly different from the “placebo-pelleted” group). These results were in agreement with those from the acute model, suggesting that the acute inhibition of supraspinal CaMKII effectively disrupted the established opioid antinociceptive tolerance.

KN93 reversed opioid dependence in a chronic model of opioid dependence

We next tested the effect of an acute CaMKII inhibition on opioid dependence in a chronic opioid dependence model. On Day 6 post morphine-pelleting, naloxone precipitated a significant number of withdrawal jumps in morphine-implanted mice when compared with placebo-pelleted mice, indicative of the presence of opioid dependence (Fig. 6). Acutely administrated KN93 (*i.c.v.*, 15 min before naloxone administration) dose-dependently decreased the number of naloxone-withdrawal jumping in morphine-pelleted mice. At the highest dose (45 nmol), KN93 completely suppressed the withdrawal jumping ($p < 0.001$ compared to the morphine-pelleted mice; not significantly different from the placebo-pelleted mice). KN93 at lower doses (5-30 nmol) also significantly reduced the number of withdrawal jumping (Fig. 6), suggesting that acute inhibition of CaMKII was effective in reversing established opioid dependence in the chronic mouse model of opioid dependence.

Downstream Effectors of CaMKII

CaMKII affects a number of downstream effectors including receptors and transcription factors. The transcriptional factor cAMP response element-binding protein (CREB), a key CaMKII downstream effector, has been previously proposed to be important in

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opioid tolerance and dependence (Nestler, 2001). We examined the activation of CREB (pCREB) in mice that have been treated with morphine. As expected, chronic treatment with morphine increased the levels of pCREB (Fig. 7).

Discussion

The current study tested the hypothesis that CaMKII can directly regulate opioid tolerance and dependence. Previous studies using a rat model of opioid tolerance and dependence (10mg/kg morphine, *s.c.*, every 12 h) demonstrated that opioid tolerance and dependence were prevented by chronically microinjecting into hippocampus chemical CaMKII inhibitors or antisense oligodeoxynucleotides (Fan et al., 1999; Lu et al., 2000). The same chronic treatments in striatum (tolerance) and amygdale (dependence) were not effective. Neither was the acute CaMKII inhibition in hippocampus able to affect opioid tolerance (Fan et al., 1999). Based on the hippocampus-specific action of CaMKII inhibition and the requirement of chronic administration, it has been suggested that CaMKII modulated opioid tolerance and dependence through the learning and memory pathways. This was not entirely surprising since opioid tolerance and dependence have been hypothesized to involve learning and memory (Siegel, 1976). Several antagonists of NMDA receptor, another member of a group of genes essential for LTP and learning and memory, have also been previously found to prevent the development of opioid tolerance and dependence (Marek et al., 1991; Trujillo and Akil, 1991).

Further studies found that the effect of MK801, an NMDA receptor antagonist, did not depend entirely on its ability to interfere with associative learning (Gutstein and Trujillo, 1993). Instead, MK801 prevented morphine antinociceptive tolerance in spinalized rats

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(Gutstein and Trujillo, 1993), indicating that the NMDA receptor directly (i.e., independent of learning and memory) affected opioid tolerance and dependence.

In this study, we tested if such a direct role existed for CaMKII. Since the acute inhibition of hippocampal CaMKII did not affect opioid tolerance (Fan et al., 1999), it would suggest that the acute inhibition of CaMKII had no or minimal impact on learning and memory. We took the study paradigm a step further to study the acute supraspinal inhibition of CaMKII in two models of opioid tolerance and dependence in mice. An effect by the acute supraspinal inhibition served as an indication for a direct effect by CaMKII.

Unlike the acute hippocampal CaMKII inhibition, the acute supraspinal CaMKII inhibition by KN93 (*i.c.v.*), a selective CaMKII inhibitor (Niki et al., 1993), effectively reversed the established opioid tolerance and dependence in an acute model of opioid tolerance/dependence. While morphine-treated groups of mice showed antinociceptive tolerance, acutely administered (15 min prior) KN93 was able to dose-dependently reverse the antinociceptive tolerance. The effect was not observed in mice acutely treated with KN92, a kinase-inactive structural analogue of KN93 (Tombes et al., 1995). Moreover, the effect of KN93 was not due to any direct effect on nociception or antinociception, as KN93 did not produce antinociception by itself or interfere with acute morphine antinociception. A previous study also did not find an interference by KN93 (*i.c.v.*) on morphine (*s.c.*)-produced antinociception or hyperlocomotion (Narita et al., 2004). The western blotting analysis, on the other hand, confirmed the inhibition of CaMKII activity by the acute *i.c.v.* administration of KN93.

A direct action of CaMKII in opioid tolerance and dependence was further supported by studies in a chronic model (1 x 75 mg morphine pellet/mouse for 6 days) of opioid tolerance and dependence in mice. The acute CaMKII inhibition by KN93 reversed the

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already-established morphine tolerance and dependence, indicating that the effect of acute CaMKII inhibition was not limited to a particular model. We have previously found that acute spinal inhibition of CaMKII reversed the established morphine tolerance in rats (Wang et al., 2003), suggesting that both spinal and supraspinal CaMKII are essential for the maintenance of opioid tolerance and dependence.

The time course of CaMKII activation correlated with the development of opioid tolerance and dependence in both models that we tested (Ho et al., 1975; Patrick et al., 1975; Bilsky et al., 1996). These data were in agreement with previous findings that CaMKII can be activated in different CNS regions after the treatment with morphine (Fan et al., 1999; Wang et al., 2003; Liang et al., 2004).

The direct action of CaMKII in opioid tolerance and dependence was supported by data from cellular studies. In cellular models, desensitization of mu opioid receptor was found to be affected by CaMKII. DAMGO-induced activation of GIRK channels was significantly desensitized in the presence of a constitutively active CaMKII and the μ opioid receptor (μ OR) in *Xenopus* oocytes (Mestek et al., 1995; Koch et al., 1997). Anatomically, a direct mechanism by CaMKII was also plausible since μ OR and CaMKII were found to co-exist in dorsal root ganglia and superficial layers of spinal dorsal horn, areas critical for pain transmission (Bruggemann et al., 2000). Moreover, upon the activation of μ OR, these receptors were internalized to co-localize with CaMKII in the cytoplasm (Bruggemann et al., 2000).

Besides opioid receptors, CaMKII may also interact with the NMDA receptors, leading to opioid tolerance and dependence. Ca^{2+} influx via the activation of NMDA receptors results in activation and autophosphorylation of CaMKII at position Thr286 (Fukunaga et al., 1992; Strack et al., 2000). Activated CaMKII, in turn, can phosphorylate and

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activate NMDA receptors, leading to more Ca^{2+} influx through the channel (Kitamura et al., 1993). Therefore, CaMKII and the NMDA receptor can interact with each other in a feed-forward fashion. As noted above, the NMDA receptor has been shown to directly affect opioid tolerance and dependence (Gutstein and Trujillo, 1993).

In addition to opioid receptors and NMDA receptors, numerous other downstream effectors can be affected by CaMKII, including transcription factors such as CREB (Sheng et al., 1991), activating transcript factor 1 (ATF-1) (Shimomura et al., 1996), serum response factor (Misra et al., 1994), and CAAT-enhancer-binding protein β (C/EBP β) (Wegner et al., 1992). We found that activation of CREB (pCREB) was closely related to the activation of CaMKII in mice that were tolerant to/dependent on morphine. These data are in agreement with previous findings that CREB is important for opioid tolerance and dependence (Nestler, 2001; Valverde et al., 2004).

In summary, our data provided the initial evidence for a critical role of CaMKII in directly promoting opioid tolerance and dependence. This mechanism is in addition to its impact on tolerance and dependence via learning and memory. Identifying such a direct mechanism will not only have a profound impact on our understanding, but also may lead to pharmacological interventions targeting the CaMKII pathway for the attenuation of opioid tolerance and dependence. Recently, we found that trifluoperazine, an orally available antipsychotic drug, was capable of disrupting opioid tolerance by inhibiting CaMKII (Tang et al., 2006). It is conceivable that CaMKII inhibitors such as phenothiazine antipsychotics should be tested in humans for the prevention and/or treatment of opioid addiction and tolerance.

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References

- Bilsky EJ, Bernstein RN, Wang Z, Sadee W, and Porreca F (1996) Effects of naloxone and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ and the protein kinase inhibitors H7 and H8 on acute morphine dependence and antinociceptive tolerance in mice. *J Pharmacol Exp Ther* 277:484-490.
- Bruggemann I, Schulz S, Wiborny D, and Holtt V (2000) Colocalization of the mu-opioid receptor and calcium/calmodulin-dependent kinase II in distinct pain-processing brain regions. *Brain Res Mol Brain Res* 85:239-250.
- Fan GH, Wang LZ, Qiu HC, Ma L, and Pei G (1999) Inhibition of calcium/calmodulin-dependent protein kinase II in rat hippocampus attenuates morphine tolerance and dependence. *Mol Pharmacol* 56:39-45.
- Fields A, and Sarne Y (1997) The stimulatory effect of opioids on cyclic AMP production in SK-N-SH cells is mediated by calcium ions. *Life Sci* 61:595-602.
- Fukunaga K, Soderling TR, and Miyamoto E (1992) Activation of Ca²⁺/calmodulin-dependent protein kinase II and protein kinase C by glutamate in cultured rat hippocampal neurons. *J Biol Chem* 267:22527-22533.
- Gutstein HB, and Trujillo KA (1993) MK-801 inhibits the development of morphine tolerance at spinal sites. *Brain Res* 626:332-334.
- Ho IK, Brase DA, Loh HH, and Way EL (1975) Influence of L-tryptophan on morphine analgesia, tolerance and physical dependence. *J Pharmacol Exp Ther* 193:35-43.
- Hudmon A, and Schulman H (2002) Structure-function of the multifunctional Ca²⁺/calmodulin-dependent protein kinase II. *Biochem J* 364:593-611.

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- Kitamura Y, Miyazaki A, Yamanaka Y, and Nomura Y (1993) Stimulatory effects of protein kinase C and calmodulin kinase II on N- methyl-D-aspartate receptor/channels in the postsynaptic density of rat brain. *J Neurochem* 61:100-109.
- Koch T, Krosiak T, Mayer P, Raulf E, and Holtt V (1997) Site mutation in the rat mu-opioid receptor demonstrates the involvement of calcium/calmodulin-dependent protein kinase II in agonist-mediated desensitization. *J Neurochem* 69:1767-1770.
- Liang D, Li X, and Clark JD (2004) Increased expression of Ca²⁺/calmodulin-dependent protein kinase II alpha during chronic morphine exposure. *Neuroscience* 123:769-775.
- Lou L, Zhou T, Wang P, and Pei G (1999) Modulation of Ca²⁺/calmodulin-dependent protein kinase II activity by acute and chronic morphine administration in rat hippocampus: differential regulation of alpha and beta isoforms. *Mol Pharmacol* 55:557-563.
- Lu L, Zeng S, Liu D, and Ceng X (2000) Inhibition of the amygdala and hippocampal calcium/calmodulin-dependent protein kinase II attenuates the dependence and relapse to morphine differently in rats. *Neurosci Lett* 291:191-195.
- Marek P, Ben-Eliyahu S, Gold M, and Liebeskind JC (1991) Excitatory amino acid antagonists (kynurenic acid and MK-801) attenuate the development of morphine tolerance in the rat. *Brain Res* 547:77-81.
- Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, and Kandel ER (1996) Control of memory formation through regulated expression of a CaMKII transgene. *Science* 274:1678-1683.
- Mestek A, Hurley JH, Bye LS, Campbell AD, Chen Y, Tian M, Liu J, Schulman H, and Yu L (1995) The human mu opioid receptor: modulation of functional desensitization by calcium/calmodulin-dependent protein kinase and protein kinase C. *J Neurosci* 15:2396-2406.

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- Misra RP, Bonni A, Miranti CK, Rivera VM, Sheng M, and Greenberg ME (1994) L-type voltage-sensitive calcium channel activation stimulates gene expression by a serum response factor-dependent pathway. *J Biol Chem* 269:25483-25493.
- Narita M, Matsumura Y, Ozaki S, Ise Y, Yajima Y, and Suzuki T (2004) Role of the calcium/calmodulin-dependent protein kinase ii (CaMKII) in the morphine-induced pharmacological effects in the mouse. *Neuroscience* 126:415-421.
- Nehmad R, Nadler H, and Simantov R (1982) Effects of acute and chronic morphine treatment of calmodulin activity of rat brain. *Mol Pharmacol* 22:389-394.
- Nestler EJ (2001) Molecular neurobiology of addiction. *Am J Addict* 10:201-217.
- Niki I, Okazaki K, Saitoh M, Niki A, Niki H, Tamagawa T, Iguchi A, and Hidaka H (1993) Presence and possible involvement of Ca/calmodulin-dependent protein kinases in insulin release from the rat pancreatic beta cell. *Biochem Biophys Res Commun* 191:255-261.
- Niu S, Kuo CH, Gan Y, Nishikawa E, Sadakata T, Ichikawa H, and Miki N (2000) Increase of calmodulin III gene expression by mu-opioid receptor stimulation in PC12 cells. *Jpn J Pharmacol* 84:412-417.
- Patrick GA, Dewey WL, Spaulding TC, and Harris LS (1975) Relationship of brain morphine levels to analgesic activity in acutely treated mice and rats and in pellet implanted mice. *J Pharmacol Exp Ther* 193:876-883.
- Quillan JM, Carlson KW, Song C, Wang D, and Sadee W (2002) Differential effects of mu-opioid receptor ligands on Ca(2+) signaling. *J Pharmacol Exp Ther* 302:1002-1012.
- Sheng M, Thompson MA, and Greenberg ME (1991) CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* 252:1427-1430.

JPET #97733

- Shimomura A, Ogawa Y, Kitani T, Fujisawa H, and Hagiwara M (1996) Calmodulin-dependent protein kinase II potentiates transcriptional activation through activating transcription factor 1 but not cAMP response element-binding protein. *J Biol Chem* 271:17957-17960.
- Siegel S (1976) Morphine analgesic tolerance: its situation specificity supports a Pavlovian conditioning model. *Science* 193:323-325.
- Silva AJ, Paylor R, Wehner JM, and Tonegawa S (1992) Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. *Science* 257:206-211.
- Smart D, Hirst RA, Hirota K, Grandy DK, and Lambert DG (1997) The effects of recombinant rat mu-opioid receptor activation in CHO cells on phospholipase C, $[Ca^{2+}]_i$ and adenylyl cyclase. *Br J Pharmacol* 120:1165-1171.
- Spencer RJ, Jin W, Thayer SA, Chakrabarti S, Law PY, and Loh HH (1997) Mobilization of Ca^{2+} from intracellular stores in transfected neuro2a cells by activation of multiple opioid receptor subtypes. *Biochem Pharmacol* 54:809-818.
- Strack S, McNeill RB, and Colbran RJ (2000) Mechanism and regulation of calcium/calmodulin-dependent protein kinase II targeting to the NR2B subunit of the N-methyl-D-aspartate receptor. *J Biol Chem* 275:23798-23806.
- Tang L, Shukla PK, and Wang ZJ (2005) Trifluoperazine, an orally available clinically used drug, disrupts opioid antinociceptive tolerance. *Neurosci Lett (in press)*, published online since 12/24/2005, PMID: 16380209.
- Tombes RM, Grant S, Westin EH, and Krystal G (1995) G1 cell cycle arrest and apoptosis are induced in NIH 3T3 cells by KN-93, an inhibitor of CaMK-II (the multifunctional Ca^{2+} /CaM kinase). *Cell Growth Differ* 6:1063-1070.

JPET #97733

Trujillo KA, and Akil H (1991) Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science* 251:85-87.

Valverde O, Mantamadiotis T, Torrecilla M, Ugedo L, Pineda J, Bleckmann S, Gass P, Kretz O, Mitchell JM, Schutz G, and Maldonado R (2004) Modulation of anxiety-like behavior and morphine dependence in CREB-deficient mice. *Neuropsychopharmacology* 29:1122-1133.

Wang Z, Bilsky EJ, Porreca F, and Sadee W (1994) Constitutive mu opioid receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. *Life Sci* 54:L339-350.

Wang Z, Gardell LR, Ossipov MH, Vanderah TW, Brennan MB, Hochgeschwender U, Hruby VJ, Malan TP, Jr., Lai J, and Porreca F (2001) Pronociceptive actions of dynorphin maintain chronic neuropathic pain. *J Neurosci* 21:1779-1786.

Wang ZJ, Tang L, and Xin L (2003) Reversal of morphine antinociceptive tolerance by acute spinal inhibition of Ca(2+)/calmodulin-dependent protein kinase II. *Eur J Pharmacol* 465:199-200.

Way EL, Loh HH, and Shen FH (1969) Simultaneous quantitative assessment of morphine tolerance and physical dependence. *J Pharmacol Exp Ther* 167:1-8.

Wegner M, Cao Z, and Rosenfeld MG (1992) Calcium-regulated phosphorylation within the leucine zipper of C/EBP beta. *Science* 256:370-373.

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Footnotes

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Legends for Figures

Figure 1. Effect of KN93 on morphine antinociceptive tolerance and CaMKII activity.

(A) *The dose-response curve of KN93 in reversing the established opioid tolerance.* Groups of 8 male ICR mice received *s.c.* injections of morphine (“MS”) or an equal volume of saline (“Saline”). Four hours later, KN93 (5, 15, or 30 nmol, dissolved in 5 μ l saline, *i.c.v.*) was given to several groups of morphine-treated mice. The remaining groups of mice received an *i.c.v.* injection of saline (5 μ l). 15 min later, morphine (1, 3, or 10 nmol, *i.c.v.*) was administrated to mice to determine its antinociception by the tail-flick assay. Data are expressed in %MPE (Mean \pm SEM).

(B) *Comparison of the effects of KN93 and KN92 on opioid antinociceptive tolerance.* In morphine (100mg/kg, *s.c.*)-treated mice, tolerance to morphine (10nmol, *i.c.v.*) was reversed by the acute treatments with KN93 (15, 30 nmol, *i.c.v.*), but not by the lowest dose of KN93 (5 nmol, *i.c.v.*) or KN92 (30 nmol, *i.c.v.*).

(C) *The time course of the activation of CaMKII by morphine.* Groups of 3 mice were *s.c.* injected with saline or morphine (100mg/kg). Brain samples were taken at the indicated time points to determine supraspinal CaMKII activity. The activated CaMKII was determined by the western blotting method using an antibody specific for the T286-phosphorylated CaMKII (pCaMKII). Histogram data, expressed in Mean \pm SEM, were constructed from the representative figure shown and 3 other experiments.

(D). *Inhibition of CaMKII activation by KN93.*

Groups of 3 morphine (100mg/kg, *s.c.*, for 4 h)-treated mice were injected *i.c.v.* with either saline (5 μ l) or KN93 (30nmol in 5 μ l saline). 15 min later, brain samples were taken for the

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analyses of supraspinal CaMKII activity as described above. Histogram data, expressed in Mean \pm SEM, were constructed from the representative figure shown and 3 other experiments. $**p<0.01$, $**p<0.01$, $***p<0.001$ compared to the “saline” group; $###p<0.001$ compared to the “MS” group.

Figure 2. Effects of KN93 and KN92 on morphine-antinociception.

Groups of 8 male ICR mice received *i.c.v.* injections of KN93 (30 nmol in 5 μ l saline), KN92 (30nmol in 5 μ l saline) or saline (5 μ l). 15 min later, morphine (1, 3, or 10 nmol, *i.c.v.*) was administrated to mice to determine its antinociception by the tail-flick assay. Neither KN93 nor KN92 altered acute morphine antinociception ($p>0.05$). Data are expressed in %MPE (Mean \pm SEM).

Figure 3. Effect of acute CaMKII inhibition by KN93 on morphine dependence.

(A) *Dose-response curve of KN93 in reversing the established opioid dependence.*

Groups of 8 male ICR mice received *s.c.* injections of morphine (“MS”) or an equal volume of saline (“Saline”). All mice received *i.p.* injections of naloxone (1, 3, or 10 mg/kg) 5 h later. 15 min before the injection of naloxone, KN93 (5, 15, or 30 nmol, dissolved in 5 μ l saline, *i.c.v.*) was given to several groups of morphine-treated mice. The remaining groups of mice received *i.c.v.* injections of saline (5 μ l). The numbers of vertical jumps were recorded for each group of mice and expressed in Mean \pm SEM.

(B) *Comparison of the effects of KN93 and KN92 on opioid dependence.* In morphine (100mg/kg, *s.c.*)-treated mice, dependence on morphine was revealed by naloxone (10mg/kg, *i.p.*)-precipitated withdrawal jumping 5 h later. The withdrawal jumping was completely

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blocked by an acute treatment with KN93 at the highest dose (30 nmol, *i.c.v.*), and attenuated by KN93 at 15 nmol ($p<0.01$). KN93 at the lowest dose (5 nmol, *i.c.v.*) or KN92 (30 nmol, *i.c.v.*) did not significantly alter morphine dependence ($p>0.05$). *** $p<0.001$ compared to the “saline” group; ## $p<0.01$, ### $p<0.001$ compared to the “MS” group.

Figure 4. Prevention of morphine antinociceptive tolerance and physical dependence by KN93.

Groups of 8 male ICR mice received KN93 (30 nmol in 5 μ l saline, *i.c.v.*, designated as “KN93+MS” group), KN92 (30 nmol in 5 μ l saline, *i.c.v.*, “KN92+MS” group), or saline (5 μ l, *i.c.v.* “MS” group) immediately prior to the administration of morphine (100mg/kg, *s.c.*). Control mice received only *s.c.* injection of saline. (A) Development of tolerance to morphine was prevented by KN93, but not by KN92. (B) Development of morphine dependence, as revealed by naloxone(10mg/kg, *i.p.*)-precipitated withdrawal jumping, was prevented by KN93, but not by KN92. Data are expressed in Mean \pm SEM. *** $p<0.001$ compared to the Control group; ## $p<0.01$ compared to the “MS” group.

Figure 5 (A) Reversal of morphine antinociceptive tolerance by KN93 in a chronic model of opioid tolerance. Groups of 6 male ICR mice were *s.c.* implanted with morphine pellets (1x75mg morphine/pellet/mouse) or placebo pellets (“PB” group) on Day 0. On Day 6, KN93 (5-45nmol, dissolved in 5 μ l saline, *i.c.v.*) was given to several groups of morphine-pelleted mice. The remaining groups of mice received an *i.c.v.* injection of saline (5 μ l). 15 min later, morphine (10 nmol, *i.c.v.*)-antinociception was determined. Data are expressed in %MPE (Mean \pm SEM). Antinociceptive tolerance to morphine was detected in mice received

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only morphine pellets (“MS”). In contrast, in morphine-pelleted mice that also received KN93, tolerance was completely reversed (45 nmol, $p > 0.01$ compared to “PB” group) or significantly attenuated (5-30 nmol). *** $p < 0.001$ compared to “PB” group; ### $p < 0.01$ compared to the “MS” group.

(B). Activation of supraspinal CaMKII by morphine.

Groups of 3 mice were *s.c.* implanted with morphine pellets (1 morphine pellet/mouse) or placebo pellets (“Day 0” group). Brain samples were taken at the indicated time points for the analyses of supraspinal CaMKII activity. The activated CaMKII was determined by the western blotting method using an antibody specific for T286-phosphorylated CaMKII (pCaMKII). Data, expressed in Mean \pm SEM, were from the representative figure shown and 3 other experiments. * $p < 0.05$ compared to Day 0.

(C). Chronic treatment with morphine increased supraspinal CaMKII expression. Groups of 3 mice were *s.c.* implanted with morphine pellets (1 morphine pellet/mouse) or placebo pellets (“Day 0” group). Brain samples were taken at the indicated time points for the analyses of supraspinal CaMKII expression. Data, expressed in Mean \pm SEM, were from the representative figure shown and 3 other experiments. * $p < 0.05$ compared to Day 0.

(D). Activation of supraspinal CaMKII by morphine normalized by the expression of total CaMKII. Experiments were performed as described in Panels B & C. Data from these panels were analyzed together to obtain the ratio of pCaMKII/total CaMKII. * $p < 0.05$ compared to Day 0.

Figure 6. Reversal of morphine dependence by KN93 in a chronic model of opioid dependence.

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Groups of 6 male ICR mice were *s.c.* implanted with morphine pellets (1 morphine pellet/mouse) or placebo pellets (“PB” group) on Day 0. On Day 6, KN93 (5-45nmol, dissolved in 5 μ l saline, *i.c.v.*) was given to several groups of morphine-pelleted mice. The remaining groups of mice received an *i.c.v.* injection of saline (5 μ l). 15 min later, opioid dependence was revealed by naloxone (10 mg/kg, *i.p.*)-induced withdrawal jumping in mice treated only with morphine pellets (“MS” group). In morphine-pelleted mice that also received KN93, naloxone-induced withdrawal jumping was completely blocked (45 nmol, $p>0.05$ compared with “PB” group) or significantly attenuated (5- 30 nmol). $**p<0.01$, $***p<0.001$ compared with the “PB” group; $\#p<0.05$, $###p<0.001$ compared with the “MS” group).

Figure 7. Activation of supraspinal CREB by morphine.

Groups of 3 mice were *s.c.* implanted with morphine pellets (1 morphine pellet/mouse) or placebo pellets (“Day 0” group). Brain samples were taken at the indicated time points for the analyses of supraspinal CREB activity. The activated CREB was determined by the western blotting method using an antibody specific for phosphorylated CREB (pCREB). Data, expressed in Mean \pm SEM, were from the representative figure shown and 3 other experiments. $*p<0.05$ compared to Day 0.

Fig 1

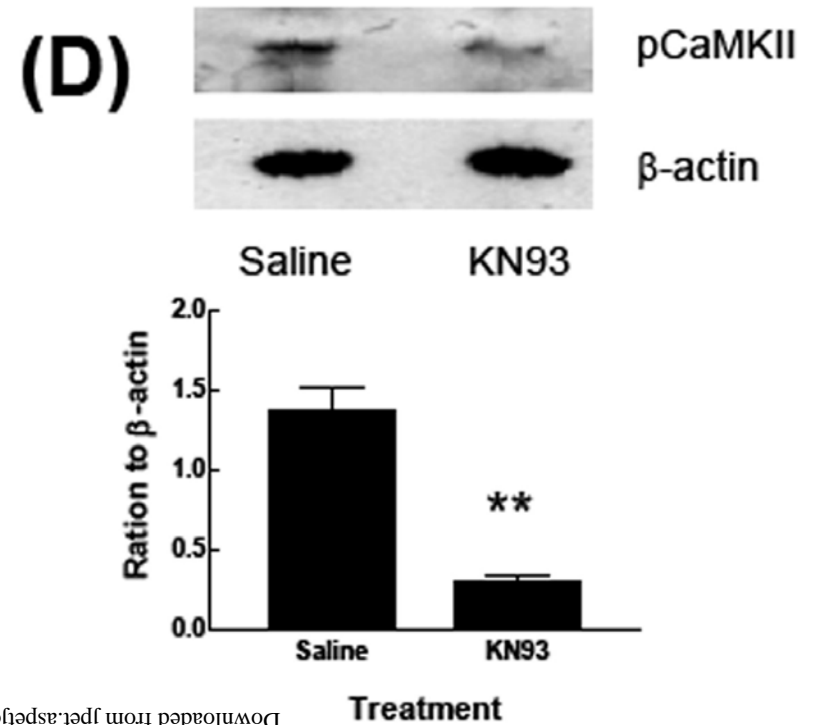
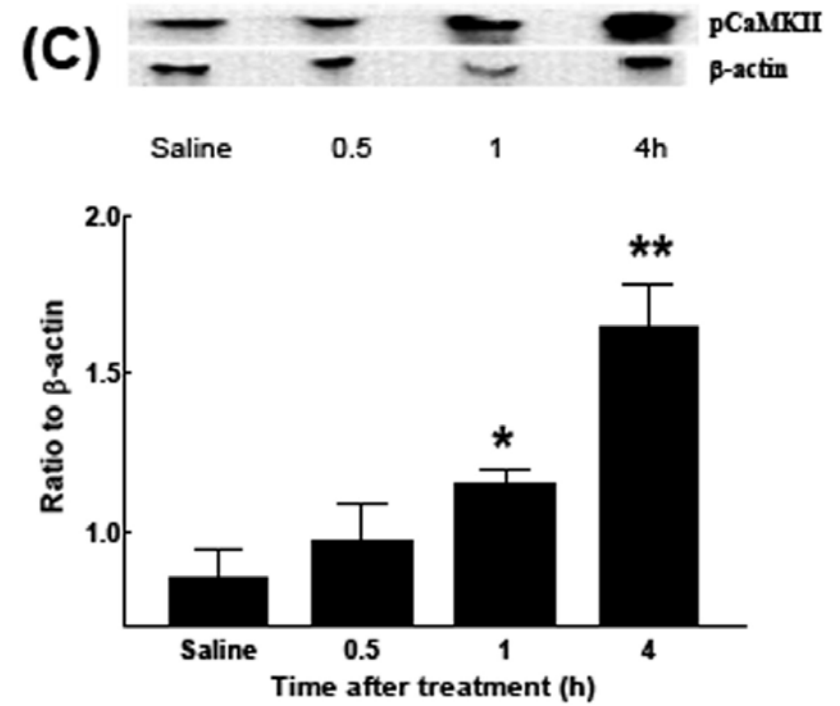
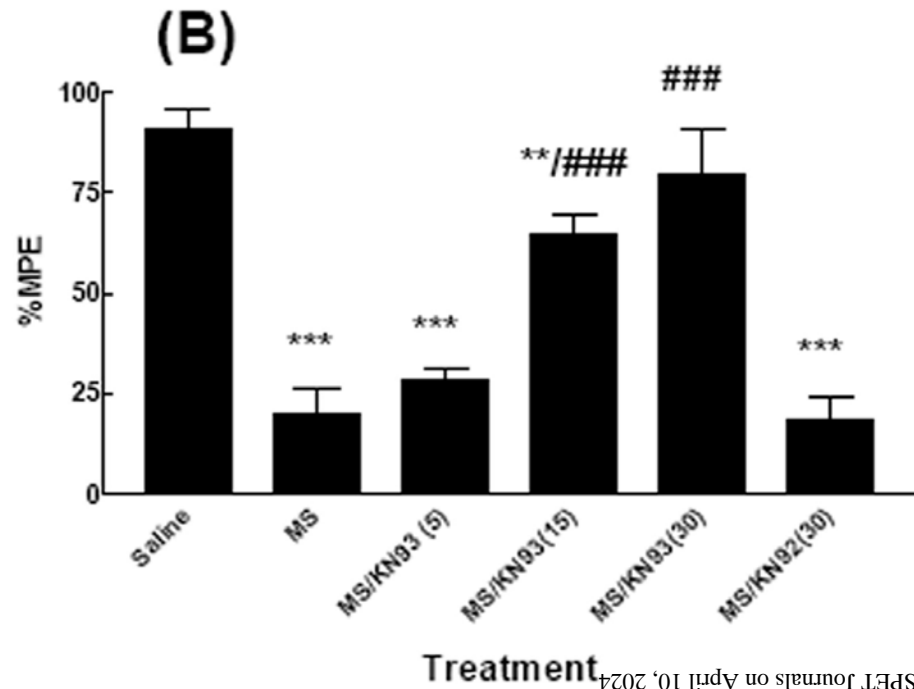
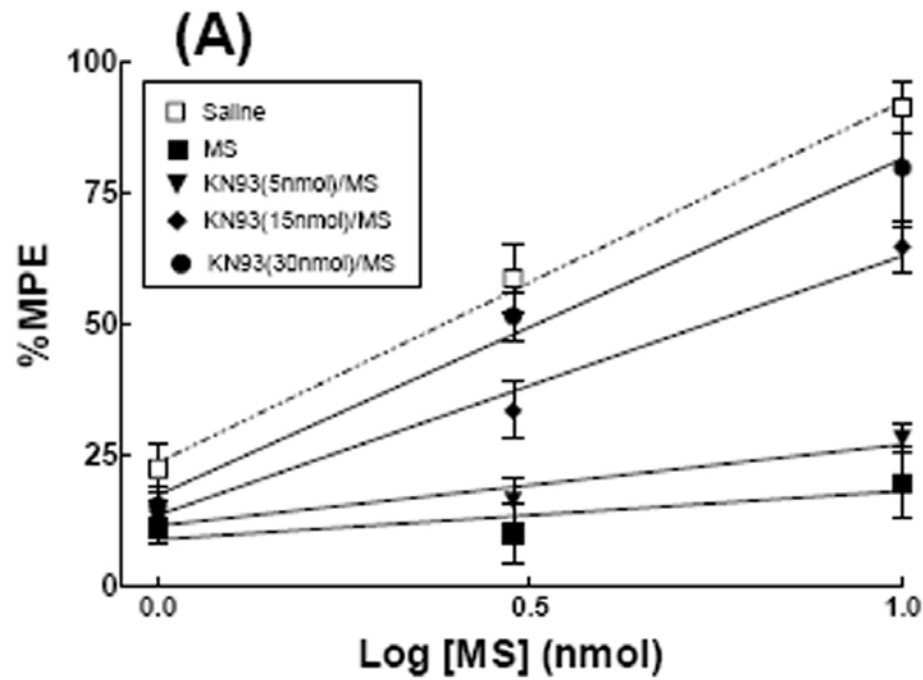
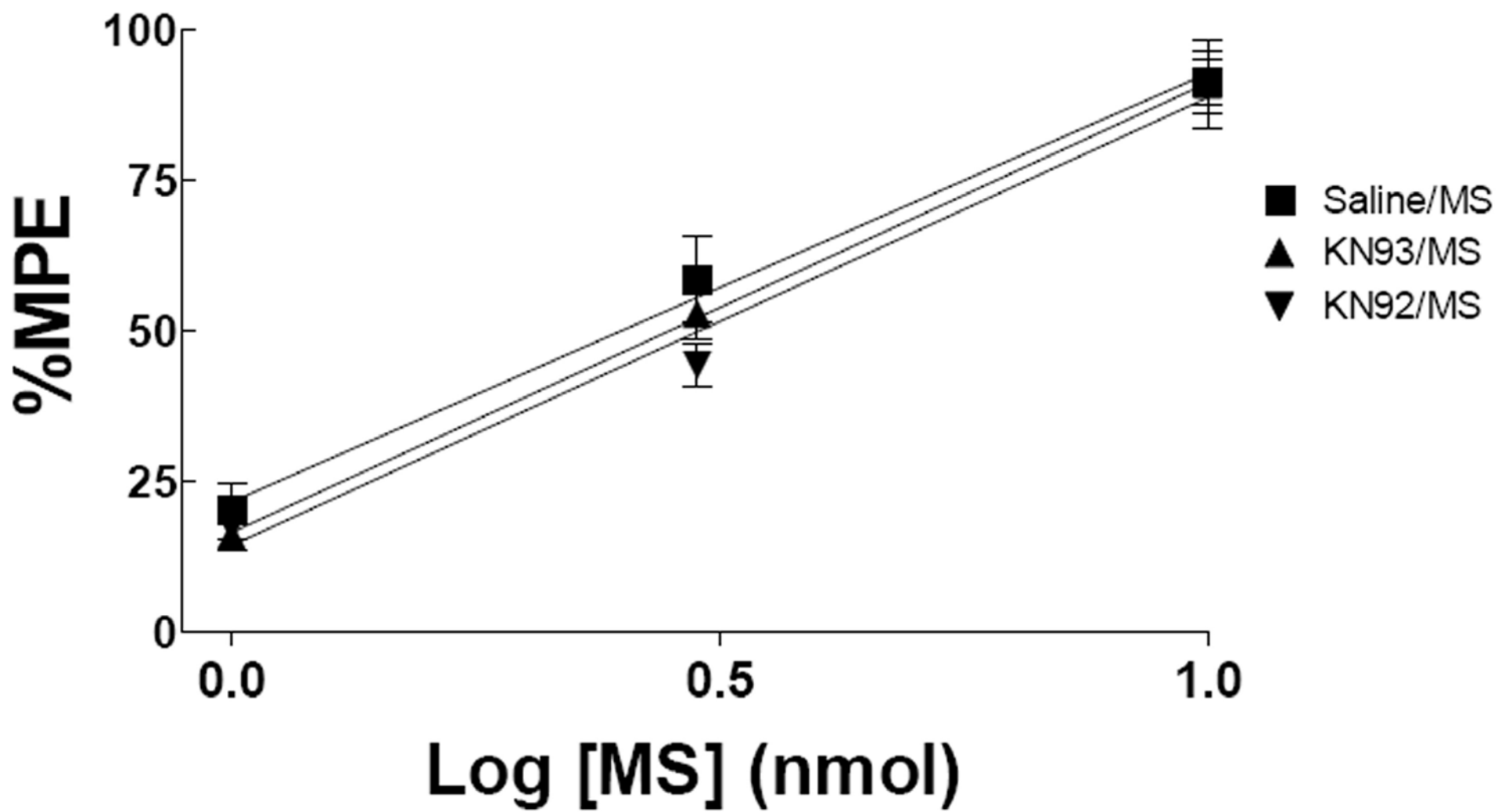


Fig 2



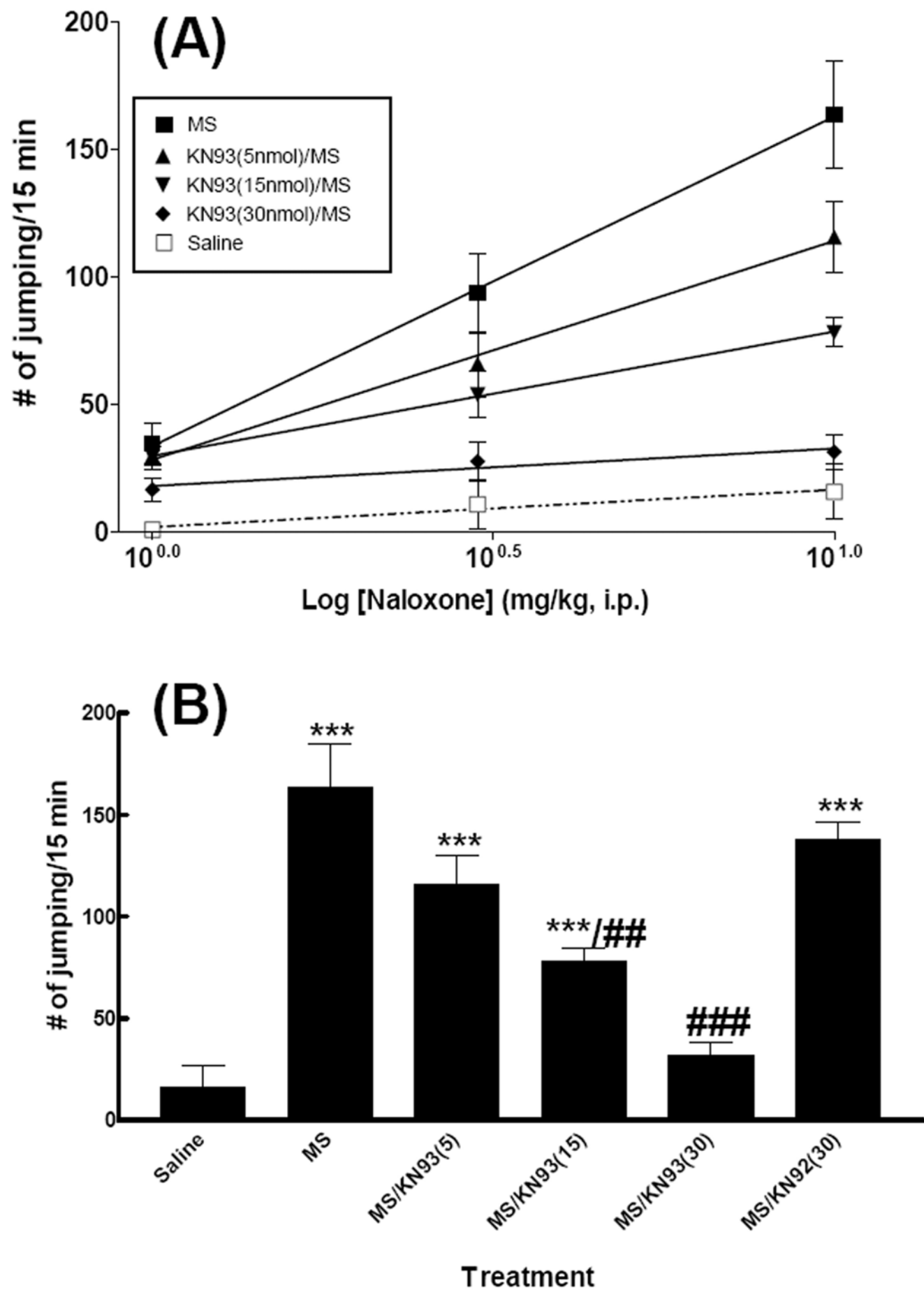
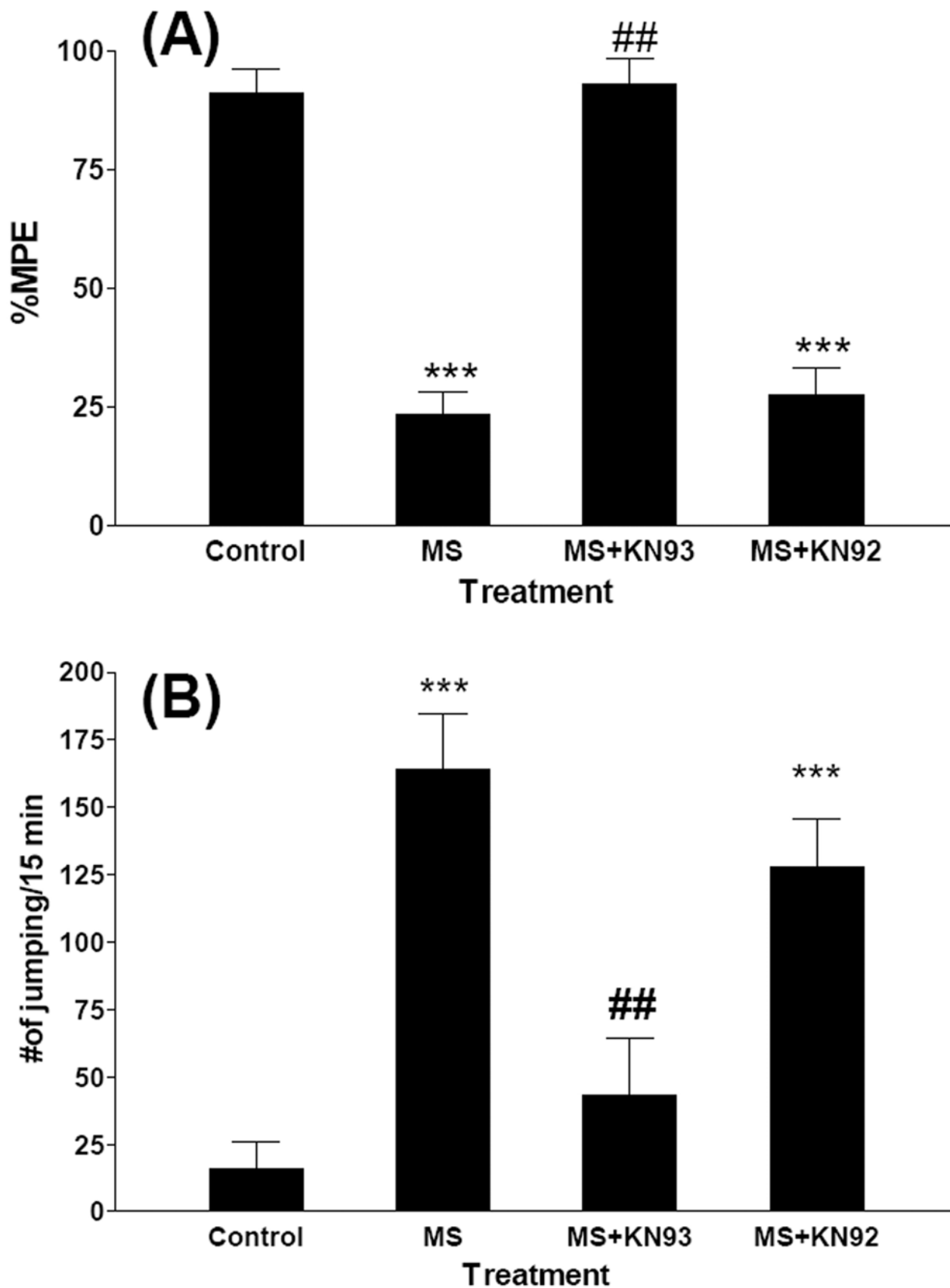
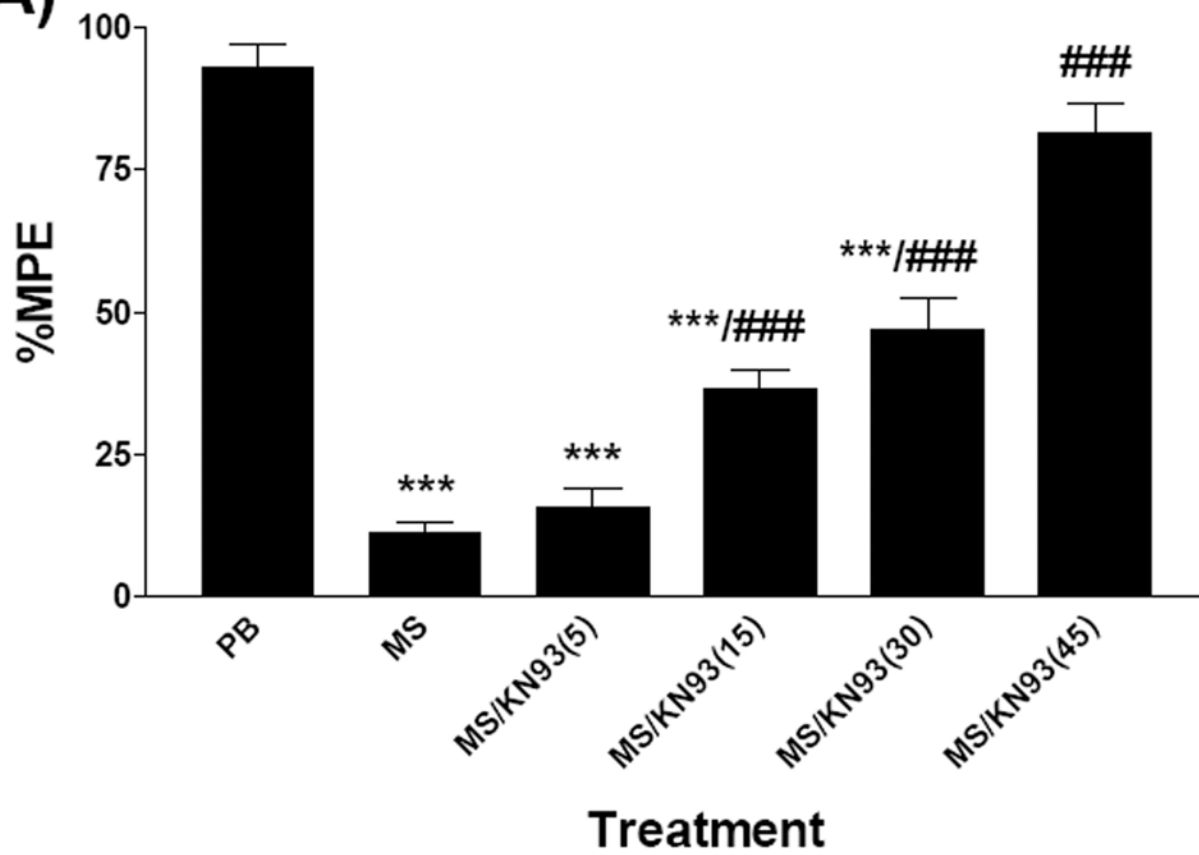


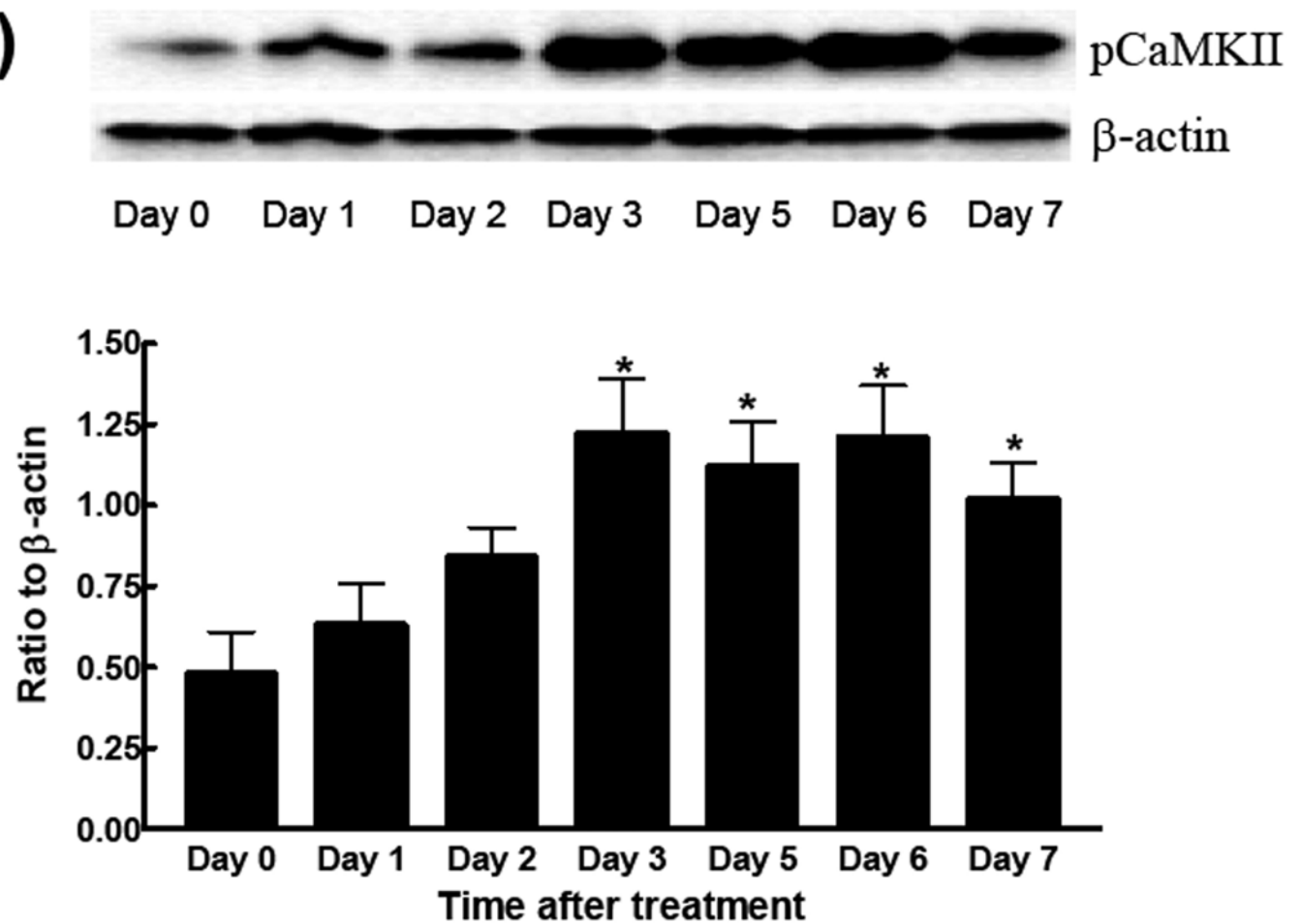
Fig 4



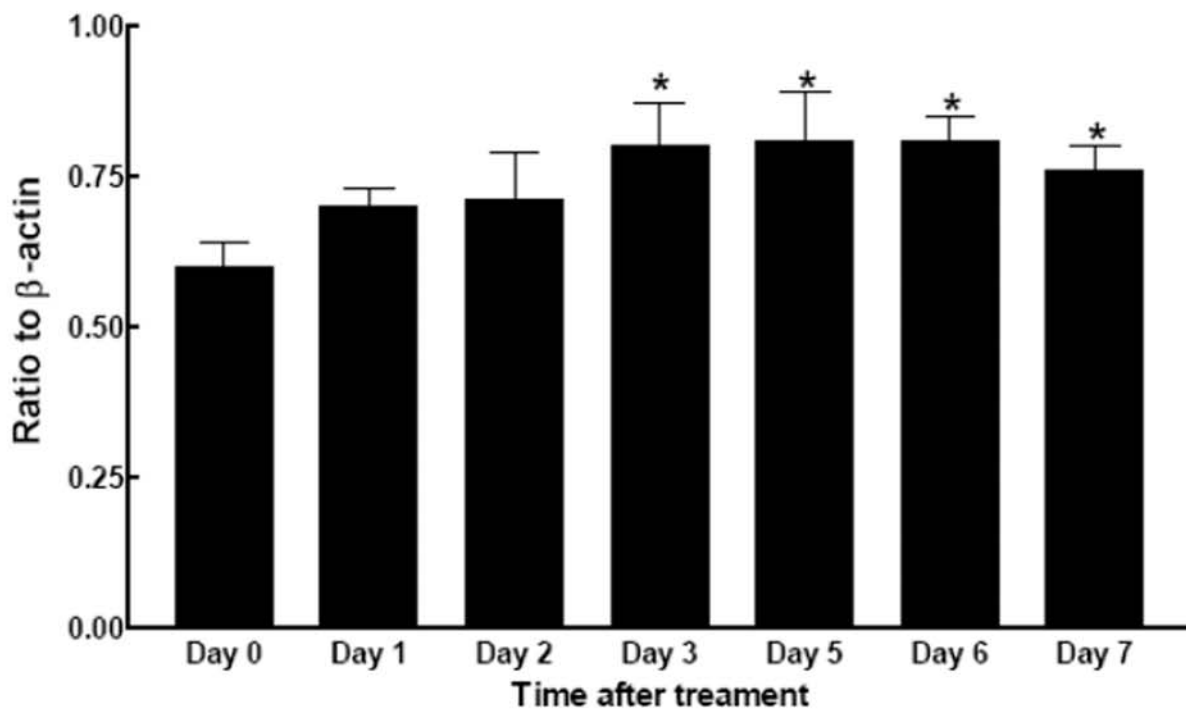
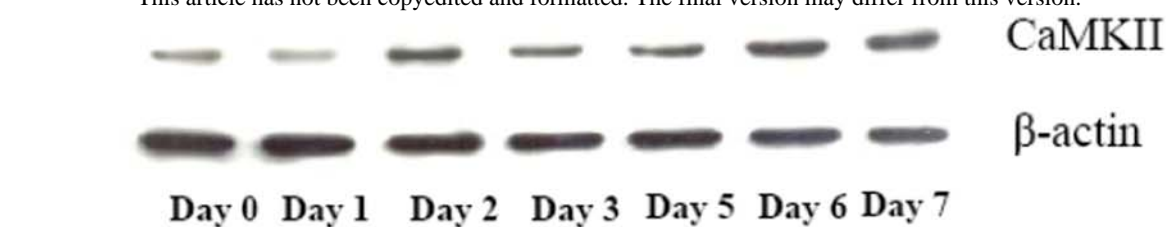
(A)



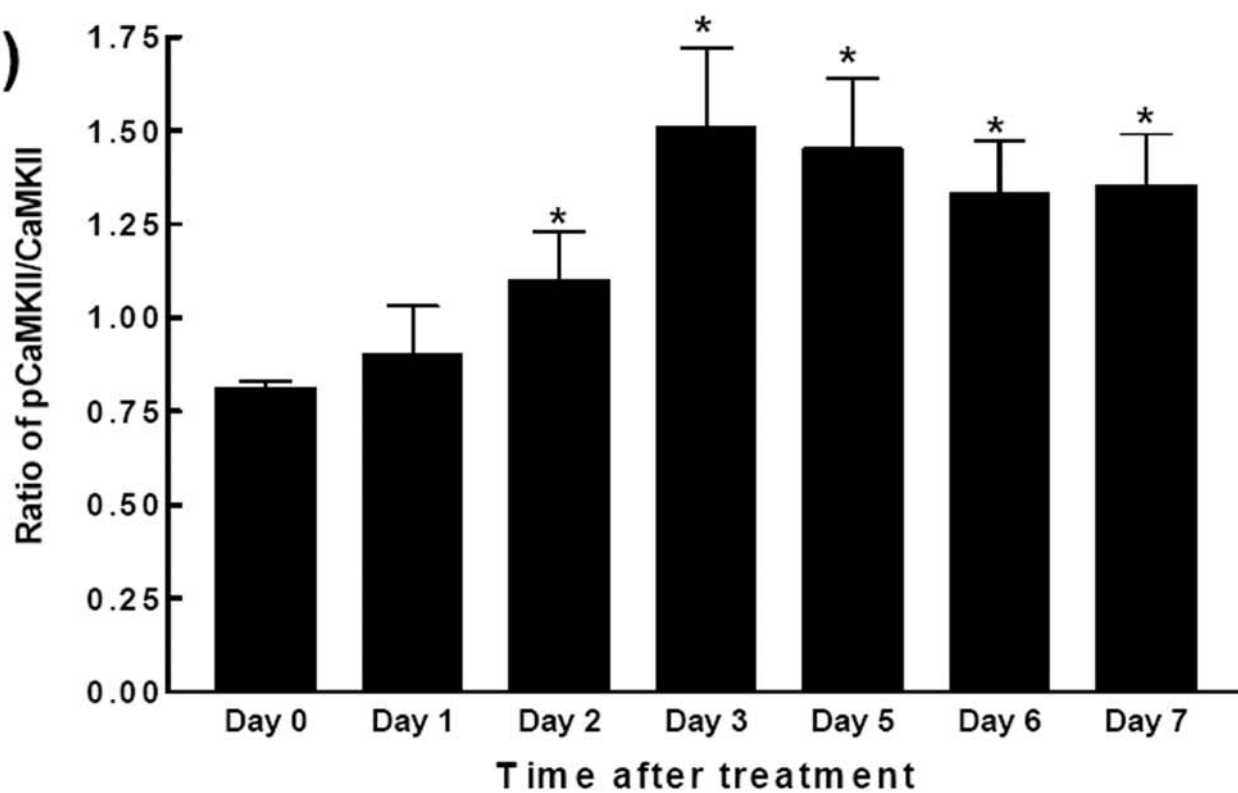
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(D)



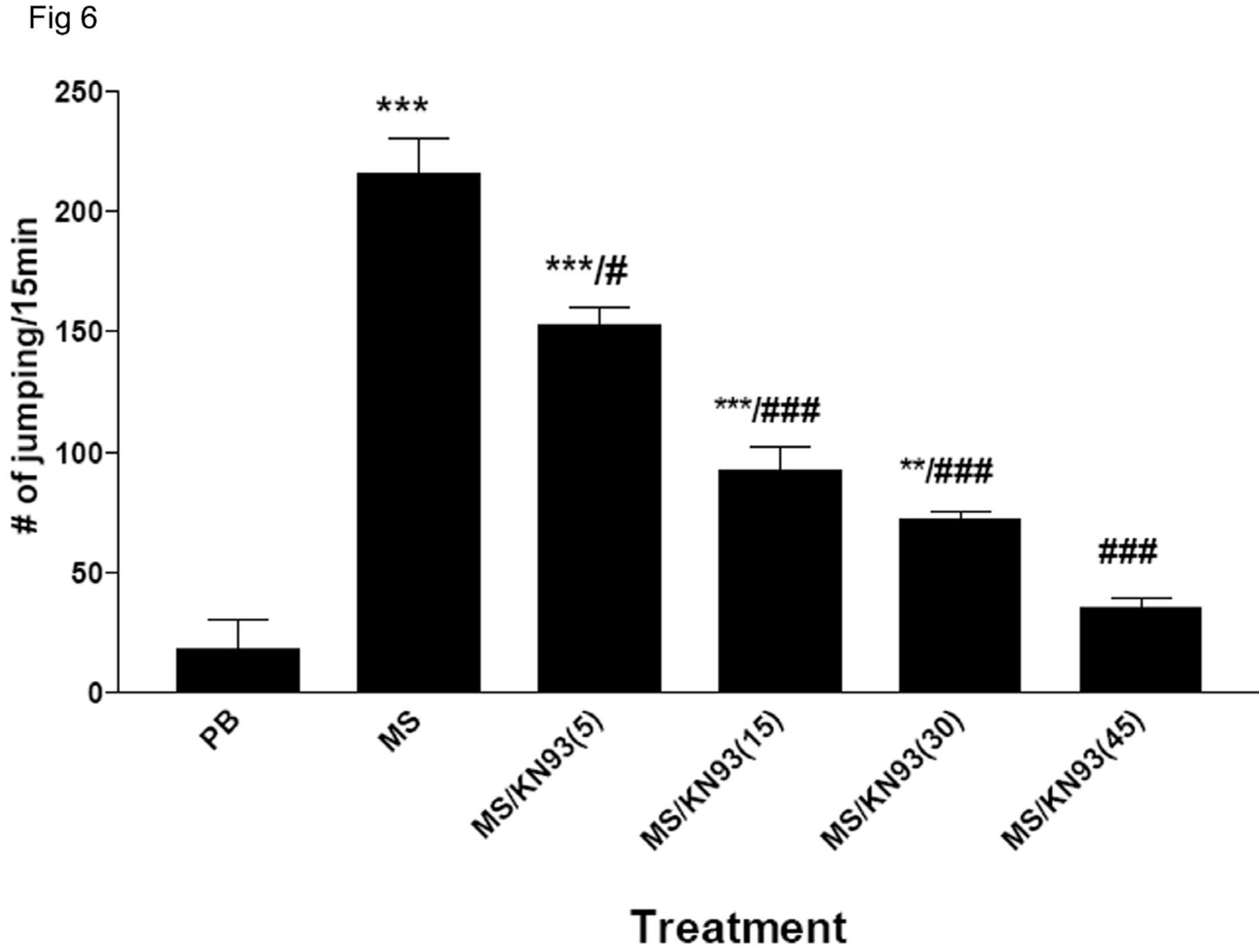


Fig 7

