JPET Fast Forward. Published on December 16, 2014 as DOI: 10.1124/jpet.114.219303
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Curcumin attenuates opioid tolerance and dependence by inhibiting CaMKIIα activity

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Running titles: Curcumin in opioid tolerance and dependence

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Manuscript information:
Figure: 8
Table: 0
Page: 32
Abstract: 221 words
Introduction: 387 words
Discussion: 1352 words
References: 36

Abbreviations:

CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; NMDA, N-methyl-D-aspartate; MPE, maximal possible effect; pCaMKII, phosphorylated CaMKII; MS, morphine.

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Abstract

Chronic use of opioid analgesics has been hindered by the development of opioid addiction and tolerance. We have reported that curcumin, a natural flavonoid from the rhizome of *Curcuma longa,* attenuated opioid tolerance, although the underlying mechanism remains unclear. In this study, we tested the hypothesis that curcumin may inhibit Ca^{2+}/calmodulin-dependent protein kinase II α (CaMKIIα), a protein kinase that have been previously proposed to be critical for opioid tolerance and dependence. In this study, we have employed a state-of-art polymeric formulation technology to produce PLGA-curcumin nanoparticles (nanocurcumin), in order to overcome the drug's poor solubility and bioavailability that have made it extremely difficult for studying in vivo pharmacological actions of curcumin. We found that PLGA-curcumin nanoparticles reduced the dose requirement by 11-33 folds. Pretreatment with PLGA-curcumin (p.o.) prevented the development of opioid tolerance and dependence in a dose dependent manner with ED_{50} of 3.9 mg/kg and 3.2 mg/kg, respectively. PLGA-curcumin dose-dependently attenuated already-established opioid tolerance (ED_{50}= 12.6 mg/kg, p.o.) and dependence (ED_{50}= 3.1 mg/kg, p.o.). Curcumin or PLGA-curcumin did not produce antinociception by itself or affect morphine (1-10 mg/kg) antinociception. Moreover, we found that the behavioral effects of curcumin on opioid tolerance and dependence correlated with its inhibition of morphine-induced CaMKIIα activation in the brain. These results suggest that curcumin may attenuate opioid tolerance and dependence by suppressing CaMKIIα activity.

Introduction

Opioid analgesics, such as morphine, have been used widely for treating moderate to severe pain. Although opioids are highly efficacious for treating acute pain, their chronic use has been hindered by the development of opioid tolerance and dependence, of which the underlying mechanisms are not fully understood (Tang *et al.*, 2006b; Wang *et al.*, 2006).

Previous work by our laboratory and others demonstrated that Ca^{2+}/calmodulin-dependent protein kinase II α (CaMKIIα) is critically important for the development and maintenance of opioid tolerance, opioid dependence, and opioid-induced hyperalgesia (Chen *et al.*, 2010). CaMKIIα is a multifunctional serine/threonine protein kinase that is abundantly expressed in the central nervous system. CaMKIIα activity in the spinal cord and brain was found to be elevated after prolonged treatment with morphine (Wang *et al.*, 2003; Liang *et al.*, 2004; Tang *et al.*, 2006a). Spinal and supraspinal inhibition of CaMKIIα was further demonstrated to be effective in preventing and reversing opioid tolerance and dependence in rodent models (Wang *et al.*, 2003; Tang *et al.*, 2006a).

Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; CAS Number 458-37-7] is a natural flavonoid component in the rhizome of *Curcuma longa* (Zingiberaceae or ginger family). A number of pharmacological effects have been reported for curcumin, including antioxidant, anti-inflammatory, chemotherapeutic, and possibly even antinociceptive effects (Asher *et al.*, 2013; Marchiani *et al.*, 2014). Several recent publications suggest that long-term treatment with curcumin is effective in attenuating opioid tolerance and dependence, though the underlying mechanism is not clear (Matsushita *et al.*, 2009; Lin *et al.*, 2010; Liang *et al.*, 2013). Interestingly,

curcumin has been recently found to inhibit the Ca^{2+}-dependent and independent kinase activities of CaMKII (Mayadevi *et al.*, 2012). We hypothesize that curcumin may attenuate opioid tolerance and dependence by inhibiting CaMKIIα in the central nervous system.

In spite of the various reported pharmacological actions, curcumin is not widely used as a therapeutic agent, likely due its relatively low solubility and bioavailability (Anand *et al.*, 2007) and lack of understanding on its mechanism of action. With the requirement of high doses in pharmacological studies and poor solubility, it is difficult to independently confirm pharmacological actions and ascertain the exact dose producing these effects. We have recently developed several polymeric nanoparticles encapsulating curcumin including PLGA-curcumin (Shen *et al.*, 2013). In this study, we have more thoroughly characterized PLGA-curcumin in two rodent models of opioid tolerance and dependence.

Materials and Methods

Materials

Morphine sulfate was purchased from Hospira (Lake Forest, IL). Curcumin, naloxone, PLGA (acid terminated; PLA:PGA 50:50 w/w; Mw 7000-17000), tetrahydrofuran (THF), trehalose, leucine, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animals

All experiment were performed after approval by the University of Illinois at Chicago Institutional Animal Care and Use Committee and complied with polices and recommendations of the International Association for the Study of Pain (IASP) and the NIH guidelines. Male ICR mice weighing 21-25g were obtained from Harlan Laboratories (Indianapolis, IN), housed in standard conditions with a light/darkness cycle (lights on at 5 a.m. and off at 7 p.m.), and provided with food and water *ad libitum* prior to experimental procedures.

Production and characterization of PLGA-curcumin nanoparticles

PLGA-curcumin nanoparticles were generated by using a multi-inlet vortex mixer (MIVM) as described (Shen *et al.*, 2013). One stream was 0.2 wt % PLGA and 0.2 wt % curcumin dissolved in THF. The other three inlet streams were deionized water as an antisolvent to precipitate the drug compound and PLGA. The volumetric flow rate of streams 1 and 2 was 6 mL/min, and it was 54 mL/min for streams 3 and 4. Freeze drying of the nanoparticle suspensions were carried out in a freeze dryer (Labconco, FreeZone 1 Liter Console Freeze Dry Systems, Kansas City, MO) at a vacuum pressure

and -47°C. Trehalose and leucine were used to prevent nanoparticle permanent aggregation during freeze drying process. PLGA-curcumin nanoparticles were homogeneously resuspended using bath sonication for 10 minutes before use. Drug loading, encapsulation efficiency of curcumin in nanoparticles, and nanoparticle size and size distributions were measured as previously described (Shen *et al.*, 2013).

Antinociception Tests

The tail-flick test was performed to assess basal nociception and morphine-induced antinociception as previously described (Tang *et al.*, 2006a; Yang *et al.*, 2011). In brief, the distal one-third of mouse-tail was immersed into a 52°C water bath, and the latency to a quick tail-flick response was recorded. Morphine-induced antinociception was determined 30 min after the injection of morphine (10mg/kg s.c.) and expressed as the percentage of maximal possible effect (MPE) according to the following formula: %MPE= 100*(postdrug latency - predrug latency)/(cut off- predrug latecy). A cut-off time of 12 sec was applied to prevent tissue damage.

Acute opioid tolerance and dependence

Mice were made acutely tolerant to and dependent on opioids by the treatment of a large dose of morphine sulfate (100mg/kg, s.c.). Morphine tolerance and dependence developed and peaked around 4 to 6 h (Shukla *et al.*, 2006; Tang *et al.*, 2006a; Yang *et al.*, 2011). An equal volume of saline was given to control mice. To assess tolerance to opioids, mice received a test dose of morphine sulfate (10mg/kg, s.c.) 4.5h later, and the antinociceptive effect was measured. The presence of tolerance to morphine was showed by a significant reduction of antinociceptive effect. Dependence to opioids was

revealed by naloxone-precipitated withdraw jumping. Mice were treated with naloxone (10mg/kg, i.p.) 5h after the injection of morphine sulfate (100mg/kg, s.c.) and were then placed in glass cylinders. Mice were observed for a 15 min period with the number of vertical jumps recorded. To prevent the development of morphine tolerance and dependence, PLGA-curcumin (2-20mg/kg, p.o.) was given 15 min before the induction dose of morphine sulfate (100mg/kg, s.c.). To reverse the established acute morphine tolerance and dependence, PLGA-curcumin (2-20mg/kg, p.o.) was given 15 min before the test dose of morphine sulfate (10mg/kg, s.c.) or naloxone.

Chronic opioid tolerance and dependence

Mice were treated with morphine sulfate (10mg/kg s.c.) twice a day for 5 consecutive days to induce opioid tolerance and dependence (Herz *et al.*, 1973; Yang *et al.*, 2011). Control mice received saline. Morphine tolerance and naloxone-precipitated withdrawal were evaluated as described above. On experimental day, PLGA-curcumin (2 - 20 mg/kg) was given by gastric gavage (p.o.) 15 min before the test dose of morphine or naloxone.

LC/MS analysis of curcumin in brain tissue

Mice were sacrificed 5min after the administration of PLGA-curcumin (20mg/kg, p.o.) or saline, and brain tissues were collected immediately. Brain tissues were homogenized with saline and extracted using liquid-liquid extraction. In brief, ethyl acetate were added to the homogenized tissue sample and mixed vigorously for 10min. The mixtures were then centrifuged at 35,000 rpm for 10min and supernatants were

collected. Vacufuge (Eppendorf, Hauppauge, NY) were used to concentrate the samples. Naive brain tissue spiked with known-amount of curcumin was used as standard. Curcumin in tissue samples was quantified by negative ion tandem mass spectroscopy, using Triple Quad LC Mass Spectrometer (Agilent, 6410QQQ, Santa Clara, CA). Identification of curcumin was performed by multiple reactions monitoring (MRM) using suitable transitions (367>216.9 m/z).

Immunoblotting analysis

Prefrontal cortex sections were quickly dissected on ice and frozen on dry ice for western blotting analysis as described previously (Luo *et al.*, 2008; Chen *et al.*, 2010). Tissues were homogenized in ice-cold RIPA buffer in the presence of protease inhibitors and phosphatase inhibitors and centrifuged. Protein content in the supernatant was determined by NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Samples (20 μg of protein) were separated by 12% SDS-polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene difluoride membrane. The membrane was preblocked using 5% bovine serum albumin (Sigma, St. Louis, MO) in 20 mM Tris-buffered saline (pH7.6) containing 0.1% Tween 20. Antibodies including a rabbit anti-(T286)pCaMKIIα antibody (1:1,000, Santa Cruz Biotechnology, Dallas, TX), a mouse anti-β-actin antibody (1:10,000; Thermo Fisher Scientific, Waltham, MA), and horseradish peroxidase-conjugated donkey anti-rabbit (for pCaMKIIα) or anti-mouse (for β-actin) secondary antibodies (1:10,000, Santa Cruz Biotechnology, Dallas, TX) were used. The specificities of the anti-(T286)pCaMKIIα antibody was characterized in transgenic mice (CaMKIIαT286A) lacking the phosphorylation site. An enhanced

chemiluminescence (ECL) detection system (Thermo Fisher Scientific) was applied for detection. ECL signals were captured by a ChemiDoc system and analyzed with the Quantity One program (Bio-Rad, Hercules, CA). Ratios of the optical densities of pCaMKIIα to those of β-actin were calculated for each sample.

Statistics

All data are presented as mean ± S.E.M. Comparisons between treatment groups were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. Statistical significance was established at 95% confidence limit.

Results

Unformulated curcumin prevented acute opioid tolerance and dependence

First, an acute model of opioid tolerance and dependence was used to investigate whether curcumin could prevent the development of opioid tolerance and dependence. To induce acute opioid tolerance, mice received a large dose of morphine sulfate (100mg/kg, s.c.), and 4.5h later, showed significantly reduced antinociception to morphine(10mg/kg) (19.1 ± 4.9% MPE, $p<0.001$), compared with MPE in the control mice pretreated with saline (91.5 ± 4.4% MPE) (Fig. 1A). Mice were treated with un formulated curcumin (20-400 mg/kg, p.o.) 15 min before the induction dose of morphine. Mice treated with curcumin (20mg/kg, p.o.) developed morphine antinociceptive tolerance (22.6 ± 5.2% MPE versus 91.5 ± 4.4% MPE in saline group, $p<0.001$) and displayed a significant number of naloxone-precipitated withdrawal jumps (82.7 ± 11.7 versus 13.0 ± 4.9 in saline group, $p<0.001$) (Fig. 1). In mice treated with curcumin (200 or 400mg/kg, p.o.), morphine (100 mg/kg) did not produce antinociceptive tolerance (75.9 ± 12.4% and 81.1 ± 7.0% MPE, n.s. from the saline-treated group, $p<0.001$ versus morphine alone) (Fig. 1). In those mice, naloxone-precipitated withdrawal jumping was significantly reduced (46.3 ± 10.8 and 37.0 ± 12.8 versus 80.4 ± 7.4 in morphine group, $p<0.05$ and $p<0.01$, respectively), suggesting that curcumin at high doses prevented the development of acute morphine tolerance and dependence (Fig. 1). The ED_{50} of curcumin is estimated to be 44.2 mg/kg (tolerance) and 109.0 mg/kg (dependence), respectively (Fig. 3).

PLGA-curcumin nanoparticles prevented acute opioid tolerance

The major problem working with curcumin was its poor solubility and bioavailability; therefore, the drug at very high doses was required in pharmacological experiments. We found that PLGA-curcumin nanoparticles significantly improved the solubility of the compound. In this study, we compared the relative potency of unformulated vs PLGA-curcumin nanoparticles in attenuating the development of opioid tolerance and dependence. Mice received PLGA-curcumin nanoparticles at three different doses (2, 6, and 20mg/kg, *p.o.*) 15 min before the induction dose of morphine. In mice receiving the highest dose of PLGA-curcumin nanoparticles (20mg/kg, *p.o.*) and morphine (100 mg/kg, *s.c.*), opioid tolerance was almost absent with largely intact morphine-induced antinociception (79.9 ± 9.8% MPE, $p<0.001$ vs. the morphine alone group; not significant vs the saline group) (Fig. 2A). PLGA-curcumin nanoparticles at 6 mg/kg also significantly attenuated morphine antinociceptive tolerance (64.5 ± 14.6% MPE, $p<0.05$ vs. the morphine alone group; not significant from the saline group) (Fig. 2A). Pretreatment with PLGA-curcumin nanoparticles at a lower dose (2mg/kg, p.o.) was ineffective in modulating opioid tolerance (32.3 ± 11.1% MPE, not significant vs the morphine alone group) (Fig. 2A). These data suggest that curcumin encapsulated in PLGA nanoparticles prevented morphine tolerance and the effect is dose-dependent with an estimated ED_{50} of 3.9 mg/kg (*p.o.*), which is 11 times lower than ED_{50} of unformulated curcumin (Fig. 3A).

PLGA-curcumin nanoparticles prevented acute opioid dependence

In these experiments, morphine (100mg/kg)- or saline-pretreated mice were challenged with naloxone (10mg/kg, *i.p.*) 5 h later. Morphine-pretreated mice exhibited

significantly higher number of naloxone-precipitated withdrawal jumps (83.6 ± 9.4) compared with that of mice received saline (14.9 ± 4.6; $p<0.001$), indicative of the development of acute dependence to morphine (Fig. 2B). In mice co-pretreated with PLGA-curcumin nanoparticles (2, 6, 20 mg/kg, p.o.) and morphine, the numbers of naloxone-precipitated withdrawal jumps were reduced to 53.5 ± 14.4, 36.3 ± 7.9 and 31.2 ± 8.5, respectively (Fig. 2B). These data indicated that curcumin encapsulated in PLGA nanoparticles dose-dependently blocked the development of acute physical dependence, with an estimated ED_{50} value of 3.2 mg/kg, which is 33 times lower than the ED_{50} of unformulated curcumin (Fig. 3B). Therefore, nanoparticles exhibited significantly improved curcumin solubility and higher potency than the unformulated curcumin.

Effect of PLGA-curcumin nanoparticles on basal nociception, morphine antinociception, and locomotor activity.

There are several potential confounding factors in interpreting the results presented in the above sections, because free or nanoencapsulated curcumin may, 1) produce antinociception; 2) interfere with morphine antinociception; 3) impair locomotor activity. Therefore, we directly determined whether free or nanoencapsulated curcumin produced antinociception or interfered with morphine-antinociception. Separate groups of mice received either nanocurcumin (20mg/kg, *p.o.*) or free curcumin (400mg/kg, *p.o.*), followed by morphine (0, 1, 3, or 10mg/kg, s.c.). Mice treated with nanocurcumin (20mg/kg, p.o.) or free curcumin (400mg/kg, p.o.) showed no difference in basal tail-flick withdrawal latencies compared with those of the saline treated mice ($p>0.05$) (Fig. 4A).

Neither free or nanocurcumin altered antinociception produced by morphine (1, 3, or 10mg/kg, s.c.) ($p>0.05$) (Fig. 4B).

We further tested the effect of curcumin and nanocurcumin on locomotor activity in a rotarod test (Chen *et al.*, 2010). Mice were placed on an accelerating rotarod, and the latencies to fall off were recorded 0.5, 1, 2 and 4 h after the administration of nanocurcumin (20mg/kg, p.o.) or free curcumin (400mg/kg, p.o.). The locomotor coordination of the mice treated with PLGA-curcumin nanoparticles or unformulated curcumin was indistinguishable from the saline-treated mice ($p>0.05$, $n=6$) (Fig. 4C).

Therefore, nanocurcumin (20mg/kg, p.o.) or curcumin (400mg/kg, p.o.) did not produce nociception, interfere with morphine antinociception, or impair locomotor activity.

PLGA-curcumin nanoparticles reversed acute opioid tolerance and dependence

We further examined whether PLGA-curcumin nanoparticles can reverse the established opioid tolerance and dependence in the acute model. Mice were given nanocurcumin (2, 6, 20 mg/kg, p.o.) 15 minutes before the test dose of morphine or naloxone. Nanocurcumin (20 mg/kg, p.o.) completely restored the morphine antinociception (86.3 ± 9.5% MPE, $p<0.001$) in morphine-pretreated mice, compared the tolerance control group (21.1 ± 4.3% MPE) (Fig. 5A). Nanocurcumin at lower dose (6 mg/kg, p.o.) produced a partial effect (45.9 ± 13.0% MPE). No effect was found in mice treated with the lowest dose (2 mg/kg) of nanocurcumin (26.5 ± 10.4% MPE, $p>0.05$) (Fig. 5A).

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PLGA-curcumin particles (20 and 6 mg/kg, p.o.) also significantly attenuated the established physical dependence to morphine, as reveled by naloxone-precipitated withdrawal jumping, resulting in 23.0 ± 8.0 ($p<0.001$) and 29.7 ± 7.2 ($p<0.01$) jumps (Fig. 5B). In comparison, naloxone produced 88.6 ± 7.6 jumps in the mice treated with morphine alone (Fig. 5B). Nanocurcumin at the lowest dose (2mg/kg, p.o.) did not have a significant effect (60.7 ± 21.7 jumps) (Fig. 5B). The ED_{50} of PLGA-curcumin nanoparticles is estimated as 12.6 mg/kg and 3.1 mg/kg for reversing the established acute morphine tolerance and dependence, respectively.

LC/MS analysis of curcumin in brain tissue after PLGA-curcumin administration

To investigate whether PLGA-curcumin can be taken up in the brain, we directly determined the level of curcumin in brain tissues after PLGA-curcumin treatment using LC/MS. Mice were treated with PLGA-curcumin (20mg/kg, p.o.) or saline, and brain tissues were collected 5min later. Brain samples from mice treated with PLGA-curcumin contained significant amount of curcumin (367>216.9 m/z transition at ~9.8min acquisition time; Fig. 6C), compared with no peak in the naive brain tissue (Fig. 6A). The naïve brain tissues spiked with known-amount of standard curcumin (8ng/g) had an identical peak (Fig. 6B). This suggests that curcumin infiltrated blood-brain barrier into brain within 5min of PLGA-curcumin administration.

PLGA-curcumin nanoparticles reduced CaMKIIα activation in acute opioid tolerance and dependence

In order to investigate the hypothesis that curcumin can inhibit CaMKIIα, which may mediate the effect seen in opioid tolerance and dependence, we determined the CaMKIIα activity in the prefrontal cortex using western blotting analysis. Activation of CaMKIIα was determined by the amount of phosphorylated CaMKIIα or pCaMKIIα. The pCaMKIIα immunoreactivity was significantly elevated in the acute opioid tolerance and dependence state. Either pretreatment or acute treatment with nanocurcumin (20 mg/kg, p.o.) significantly reduced pCaMKIIα in mice that are dependent on/tolerant to morphine (Fig. 7). These data suggest that PLGA-curcumin nanoparticles may attenuate acute opioid tolerance and dependence by inhibiting CaMKIIα activity.

PLGA-curcumin nanoparticles attenuate opioid tolerance and dependence in a chronic model

To eliminate the possibility that the promising effect of PLGA-curcumin nanoparticles was limited to acute morphine tolerance and dependence, we next studied effects of PLGA-curcumin nanoparticles in a chronic model of opioid tolerance and dependence. Mice developed antinociceptive tolerance to and physical dependence on opioids within days after receiving twice daily injections of morphine (10 mg/kg/injection, s.c.) (Herz *et al.*, 1973; Yang *et al.*, 2011). On day 6, morphine (10mg/kg, s.c.) antinociception was significantly reduced in mice chronically treated with morphine (14.2 ± 4.2% MPE, $p<0.001$) compared with that in saline-treated mice (97.6 ± 2.4% MPE) (Fig. 8A). Administration of PLGA-curcumin nanoparticles (20 mg/kg, p.o.) significantly attenuated the established chronic morphine tolerance (77.9 ± 8.8% MPE, $p<0.001$ versus the morphine group), while nanocurcumin at lower doses (6 and 2

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mg/kg, p.o.) showed marginal effects on morphine tolerance (43.5 ± 17.9% and 17.6 ± 16.0% MPE, $p>0.05$ from morphine alone) (Fig. 8A).

Nanocurcumin (20 and 6 mg/kg, p.o.) significantly blocked naloxone-precipitated withdrawal jumping (28.2 ± 7.7, $p<0.001$ and 42.5 ± 10.4, $p<0.01$ compared with the morphine group), although at the lowest dose (2 mg/kg) it was not effective (72.2 ± 6.5, $p>0.05$) (Fig. 8B). Therefore, PLGA-curcumin nanoparticles dose-dependently attenuated chronic morphine tolerance and dependence, with estimated ED_{50} of 7.2 mg/kg and 4.0 mg/kg, respectively.

PLGA-curcumin nanoparticles attenuated morphine-induced activation of CaMKIIα in the chronic model of opioid tolerance and dependence

To correlate the effect of PLGA-curcumin nanoparticles in chronic opioid tolerance and dependence with the activity of CaMKIIα, we have testified the pCaMKIIα immunoreactivity in these mice. Chronic treatment with morphine significantly enhanced the activity of CaMKIIα in the prefrontal cortex (Fig. 8C). PLGA-curcumin nanoparticles (20 mg/kg, p.o.) significantly reduced morphine-induced pCaMKIIα immunoreactivity in these mice (Fig. 8C), suggesting that the pharmacological effect of PLGA-curcumin nanoparticles in attenuating chronic opioid tolerance and dependence correlated with its ability of inhibiting CaMKIIα activity. Since CaMKIIα has been implicated as a critical regulator of opioid tolerance and dependence, our data suggest that PLGA-curcumin nanoparticles attenuated opioid tolerance and dependence by inhibiting CaMKIIα.

Discussion

In the current study, we investigated the effects of nanocurcumin at three different doses (2, 6, 20mg/kg, p.o.) in two mouse models of morphine tolerance and dependence, comparing the effects with free curcumin at three much higher doses (20, 200, 400mg/kg, p.o.). Approximately nano-encapsulation of curcumin lowered the ED_{50} by 11 (tolerance) to 33 (dependence) times.

We demonstrated that PLGA-curcumin nanoparticles not only prevented but also reversed opioid antinociceptive tolerance and physical dependence in mice. Since curcumin (up to 400mg/kg) or nanocurcumin (up to 20mg/kg) did not by itself produce antinociception, interfere with morphine antinociception, or impair locomotor activity, we concluded that the action of free or nanoencapsulated curcumin was due to its interactions with endogenous mechanisms that are important for promoting and maintaining opioid tolerance and dependence.

We further demonstrated that the behavioral effect of PLGA-curcumin nanoparticles correlated with its inhibition of CaMKIIα activity in the central nervous system. These data are consistent with the findings from our previous studies that have implicated a role for CaMKIIα in the development and maintenance of opioid tolerance and dependence (Wang *et al.*, 2003; Tang *et al.*, 2006a). CaMKIIα is co-localized with the μ opioid receptor in the anatomical areas that are critical for pain processing such as the superficial layer of the spinal dorsal horn and the dorsal root ganglia (Bruggemann *et al.*, 2000). It has been reported that constitutively active CaMKIIα increased agonist-induced desensitization of the μ opioid receptor in cellular studies (Koch *et al.*, 1997)

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and phosphorylation of the μ opioid receptor by co-localized CaMKIIα may contribute to the development of opioid tolerance and dependence (Bruggemann *et al.*, 2000).

CaMKIIα may interact with the NMDA receptors in the initiation process of opioid tolerance and dependence (Trujillo *et al.*, 1991; Gutstein *et al.*, 1993). Ca^{2+} infux through the activation of the NMDA receptor may lead to CaMKIIα autophosphorylation at Thr286 and resultant full activation of the kinase (Strack *et al.*, 2000). In turn, activated CaMKIIα can phosphorylate and activate the NMDA receptor, forming to a positive feed-forward loop between CaMKIIα and the NMDA receptor (Kitamura *et al.*, 1993; McGlade-McCulloh *et al.*, 1993).

Although the beneficial actions of inhibiting the NMDA receptor (Trujillo *et al.*, 1991; Gutstein *et al.*, 1993) or CaMKIIα (Wang *et al.*, 2003; Tang *et al.*, 2006a; Chen *et al.*, 2009) in opioid tolerance, dependence, and in chronic pain have been unequivocally demonstrated and replicated by independent studies, little advance has been made in translating these findings to the clinical use. In almost all attempts, drug toxicity was cited as a culprit for lack of success in moving these drugs to the human use. We have taken an approach in drug repurposing whereby clinically used drugs were screened and studied for inhibiting CaMKIIα. The goal is to identifying clinically used drugs with CaMKIIα inhibitory activity for alleviating problems associated with opioids, such as opioid tolerance, opioid dependence, and opioid-induced hyperalgesia (Chen *et al.*, 2010). Here, we took another approach in identifying the pharmacological mechanisms and efficacy of a traditionally used, orally effective, relatively safe botanical ingredient that is not found in the US pharmacopoeia. Moreover, we presented a method to improve compound drugability by formulating polymeric nanoparticles.

In this study, we have demonstrated that one time administration of PLGA-curcumin (20mg/kg , p.o.) is effective in preventing acute morphine tolerance and dependence, and in reversing both established acute and chronic morphine tolerance and dependence. It has been reported in the literature that chronic treatment of curcumin at the dose of 100mg/kg p.o. or 50mg/kg i.p. effectively attenuated chronic morphine tolerance and dependence (Matsushita *et al.*, 2009; Liang *et al.*, 2013). However, another group reported that chronic curcumin treatments at low doses (25mg/kg, i.p.) attenuated morphine tolerance, but high doses of curcumin (400mg/kg, i.p.) aggravated morphine tolerance (Lin *et al.*, 2010). Since curcumin has a poor solubility and bioavailability, the actual doses of curcumin exhibiting the effect may not be calculated precisely in those studies. We used curcumin encapsulated in PLGA, which greatly improves the solubility and bioavailability of curcumin, and we found that significantly lower doses of curcumin was needed to generate the comparable pharmacological effects as evidenced by the dramatic left-shift in dose-response curves.

This is our first study aiming to establish the mechanism of curcumin and efficacy of PLGA-curcumin. We correlated curcumin's effect on morphine tolerance and dependence with the significantly reduced supraspinal CaMKIIα phosphorylation. Furthermore, we demonstrated using the LC/MS analysis that curcumin, administered in PLGA-curcumin constructs, was able to infiltrate the blood-brain-barrier and become available in the brain within 5 min after its administration. In addition to the brain, curcumin may also inhibit CaMKIIα in the spinal cord. Our preliminary data indicated that curcumin attenuated opioid-induced hyperalgesia by modulating spinal CaMKIIα

activation (data not shown). Therefore, the brain may not be the only site of action for curcumin in attenuating CaMKIIα activity and opioid tolerance and dependence.

Although the current study was not designed to determine the pharmacokinetic profiles of PLGA-curcumin beyond the one point LC/MS analysis, it has been reported that the $t_{½}$ of curcumin is around 1.45h in rodents (Anand *et al.*, 2007). In our experiments, acute action was tested within an hour after PLGA-curcumin and we found it to be highly efficacious, so $t_{½}$ does not appear to be a problem. In the pretreatment, PLGA-curcumin or curcumin was given immediately before morphine and we recorded a powerful effect of these interventions in preventing the development of opioid tolerance/dependence, therefore, the presence of curcumin during the early time was sufficient to block tolerance to and dependence on morphine.

Curcumin is commonly used in traditional Asian cuisine, and its reported bioactivity profile includes antioxidant, anti-inflammatory, chemotherapeutic and neuroprotective actions. It has been suggested that curcumin abolished morphine analgesic tolerance along with the morphine-induced upregulation of BDNF transcription (Matsushita *et al.*, 2009). Liang et al. reported that daily administration of curcumin reduced opioid-induced hyperalgesia, tolerance, and physical dependence, possibly by inhibiting histone acetyltransferanse (HAT) (Liang *et al.*, 2013). On the contrary, other studies found that curcumin inhibited histone deacetylase (HDAC) (Liu *et al.*, 2005; Chen *et al.*, 2007; Lee *et al.*, 2010; Chen *et al.*, 2013). It has also been suggested that curcumin blocked corticosterone-induced phosphorylation of CaMKII in cultured hippocampal neurons (Xu *et al.*, 2009) and inhibit CaMKII autophosphorylation in vitro (Mayadevi *et al.*, 2012), though such a mechanism has not been studied in vivo. Since

we have previously found a critical role of CaMKIIα in opioid tolerance and dependence, we tested the hypothesis that curcumin's inhibitory action on CaMKIIα may be a mechanism attenuating the initiation or maintenance of opioid tolerance and dependence. Indeed, we found that curcumin was highly efficacious in inhibiting CaMKIIα in mice that were made tolerance to and dependent on morphine

In addition to opioid tolerance and dependence, curcumin has been reported to attenuate hyperalgesia in mice with chronic pain including nerve injury induced neuropathic pain (Zhao *et al.*, 2012) and diabetic neuropathic pain (Sharma *et al.*, 2006; Banafshe *et al.*, 2014). Although neuropathic pain and opioid tolerance and dependence are distinct CNS processes, they could potentially share a common contributor -- synaptic long-term potentiation -- for which CaMKIIα is required (Xin *et al.*, 2003; Lisman *et al.*, 2012). Indeed, CaMKIIα has been found to be essential and required for chronic inflammatory pain (Luo *et al.*, 2008), nerve injury-induced neuropathic pain (Chen *et al.*, 2009), and opioid-induced hyperalgesia (Chen *et al.*, 2010).

In summary, our study demonstrated that PLGA-curcumin nanoparticles, at relatively low doses, prevented and reversed opioid antinociceptive tolerance and physical dependence, correlating with its inhibitory actions on supraspinal CaMKIIα. PLGA-curcumin is significantly more potent than unformulated curcumin. Moreover, its high solubility allows us to obtain the precise doses that are required to produce these pharmacological effects. These data provide a plausible molecular mechanism for the action of curcumin in in vivo preclinical models of opioid tolerance and dependence. The rationale design, formulation, and characterization of stable PLGA-curcumin nanoparticles further not only provide a pharmacological reagent, but more importantly

can be further developed for blocking opioid dependence and for improving chronic pain therapies by attenuating tolerance to opioid drugs.

Authorship Contributions

Participated in research design: Hu, Liu, Wang

Conducted experiments: Hu, Huang, Szymusiak

Performed data analysis: Hu, Wang

Wrote or contributed to the writing of the manuscript: Hu, Liu, Wang

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Footnotes

This work was supported in part by a grant (K07 AT003647) from the National Center for Complementary and Alternative Medicine (NCCAM), National Institutes of Health (NIH). Nanoparticle formation was supported by UIC Proof of Concept (POC) award. Mechanistic CaMKII study received funds from from NSFc (81328009). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NCCAM or NIH. The final peer-reviewed manuscript is subject to the NIH Public Access Policy.

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Figure Legends

Fig. 1 Prevention of acute opioid tolerance (A) and dependence (B) by curcumin at high doses.

Separated groups of six mice were pretreated with curcumin (20, 200, 400mg/kg, p.o.) or saline, before the treatment with morphine sulfate (100mg/kg, s.c.) or saline to induce acute opioid tolerance and dependence. Curcumin (200, 400mg/kg) significantly attenuated opioid antinociceptive tolerance (A) and physical dependence (B), while it was not effective at 20mg/kg. Data are expressed in mean ± S.E.M. ***, $p<0.001$ compared with the saline group; #, $p<0.05$; ###, $p<0.001$ compared with the morphine (MS) group.

Fig. 2 Prevention of acute opioid tolerance (A) and dependence (B) by PLGA-curcumin nanoparticles.

Separate groups of eight mice were pretreated with PLGA-curcumin nanoparticles (2-20mg/kg, p.o.) or saline for 15min. Acute opioid tolerance and dependence were established by a treatment with morphine sulfate (100mg/kg, s.c.) or saline for 4.5h. PLGA-curcumin nanoparticles (6, 20mg/kg, p.o.) significantly prevented the development of opioid tolerance (A) and dependence (B) in a dose-dependence manner. Data are expressed in mean ± S.E.M. ***, $p<0.001$ compared with the saline group; #, $p<0.05$; ###, $p<0.001$ compared with the morphine (MS) group.

Fig. 3 Dose response curve of unformulated curcumin and PLGA-curcumin nanoparticles.

Dose response curve for the effects of unformulated curcumin and PLGA-curcumin nanoparticles on the acute (A) morphine tolerance and (B) dependence were plotted on a log dose scale. ED_{50} were calculated based on the dose response curve. PLGA-curcumin nanoparticles left-shifted the dose response curve and showed higher potency than unconjugated curcumin in preventing both acute morphine tolerance and dependence.

Fig. 4 Effect of PLGA-curcumin nanoparticles on basal nociception, morphine antinociception, and locomotor activity.

(A) Separate groups of six mice received (p.o.) PLGA-curcumin nanoparticles (20mg/kg), curcumin (400mg/kg) or saline. The tail-flick test was performed 1, 2, and 4 hours after the drug administration. Neither PLGA-curcumin nanoparticles (20mg/kg) nor curcumin (400mg/kg) changed the basal nociception (A) in the mice.

(B) Separate groups of six mice received (p.o.) PLGA-curcumin nanoparticles (20mg/kg), curcumin (400mg/kg) or saline, followed by morphine sulfate (1-10mg/kg, s.c.) 15min later. PLGA-curcumin nanoparticles or curcumin did not interfere with antinociceptive effects produced by morphine.

(C) Effects of PLGA-curcumin nanoparticles and curcumin on locomotor activity were assessed by the rotarod test in separate groups of 6 mice. PLGA-curcumin nanoparticles (20mg/kg, p.o.) or curcumin (400 mg/kg, p.o.) did not affect the locomotor activities.

Fig. 5 Reversal of acute opioid tolerance (A) and dependence (B) by PLGA-curcumin nanoparticles.

Groups of eight mice received morphine sulfate (100mg/kg, s.c.) to induce acute opioid tolerance and dependence. PLGA-curcumin nanoparticles (2-20mg/kg, p.o.) or saline were administered 15min before a test dose of morphine (10mg/kg, s.c.) or naloxone (10mg/kg, i.p.). PLGA-curcumin nanoparticles dose-dependently reversed the acute opioid tolerance (A) and opioid dependence (B). Data are expressed in mean ± 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) group.

Fig. 6 LC/MS analysis of curcumin in brain tissue after PLGA-curcumin administration

Mice were treated with PLGA-curcumin (20mg/kg, p.o.) or saline, and brain tissues were collected 5min later. Blank brain tissue has no peak (A), while standard curcumin added in blank brain tissue peaked around 9.8min of acquisition time (B). Brain samples from mice treated with PLGA-curcumin have a peak of curcumin at 9.8min (C).

Fig. 7 Pretreatment (A) or acute treatment (B) with PLGA-curcumin nanoparticles inhibited supraspinal CaMKIIα activity

Prefrontal cortex samples were taken to determine CaMKIIα activity. (A) For preventing acute opioid tolerance and dependence, mice were pretreated with PLGA-curcumin nanoparticles (20mg/kg, p.o.) or saline 15min before the large dose of morphine (100mg/kg, s.c.). Mice were sacrificed and prefrontal cortex samples were collected

4.5h later. Supraspinal CaMKIIα activity was significantly enhanced in morphine-treated mice. PLGA-curcumin nanoparticles (20mg/kg, p.o.) effectively prevented the elevation of CaMKIIα activity. (B) For reversing acute opioid tolerance and dependence, mice were treated the large dose of morphine (100mg/kg, s.c.). After 4.5h, mice received PLGA-curcumin nanoparticles (20mg/kg, p.o.) or saline 15 min before the tissue collection. PLGA-curcumin nanoparticles (20mg/kg, p.o.) significantly reduced the activation of CaMKIIα in acute opioid tolerant and dependent mice. Densitometry ratio (arbitrary unit) over actin was first calculated, and was further normalized to that of control (fixed to 1). Final data are expressed in mean ± S.E.M. **, p<0.01; ***, p<0.001 compared with the saline group; ##, p<0.01 compared with the morphine (MS) group.

Fig. 8 Reversal of chronic opioid tolerance (A), dependence (B), and supraspinal CaMKIIα activation (C) by PLGA-curcumin nanoparticles.

Separate group of six mice were treated with morphine sulfate (10mg/kg, s.c.) twice a day for five consecutive days to induce chronic opioid tolerance and dependence. Mice received PLGA-curcumin nanoparticles (2-20mg/kg, p.o.) or saline 15 min before a test dose of morphine (10mg/kg, s.c.) or immediately before naloxone (10mg/kg, i.p.) on day 6. Established chronic opioid tolerance (A) and dependence (B) were significantly reversed by PLGA-curcumin in a dose-dependent manner. (C) Chronic morphine exposure significantly increased the phosphorylation of CaMKIIα (pCaMKIIα), which was attenuated by acute treatment with PLGA-curcumin nanoparticles (20mg/kg, p.o.). Densitometry ratio (arbitrary unit) over actin was first calculated, and was further normalized to that of control (fixed to 1). Final data are expressed in mean ± S.E.M. **,

p<0.01; ***, p<0.001 compared with the saline group; ##, p<0.01; ###, p<0.001 compared with the morphine (MS) group.

Fig. 1

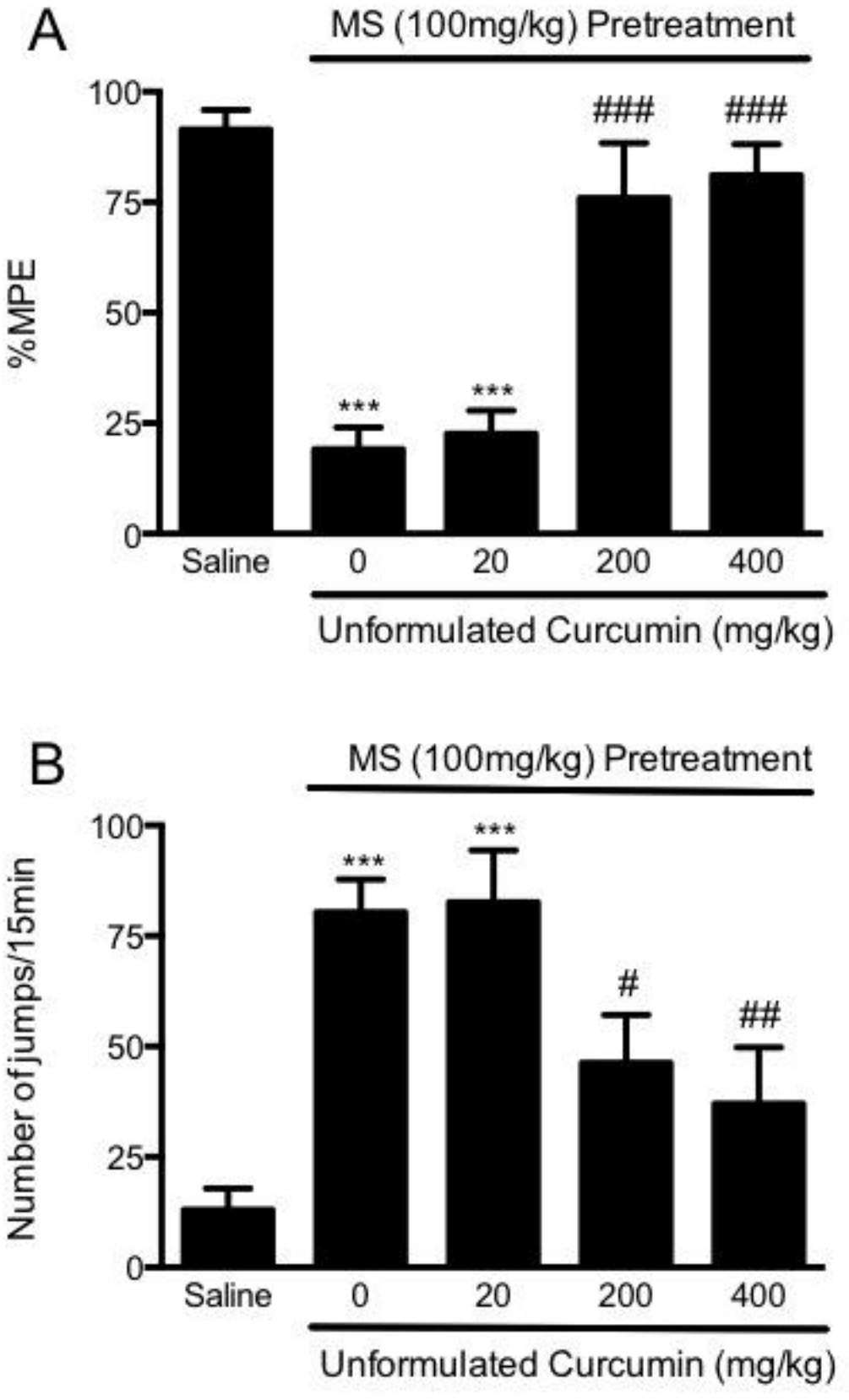

A
MS (100mg/kg) Pretreatment
%MPE
100
75
50
25
0
***
***
###
###
Saline
0
20
200
400
Unformulated Curcumin (mg/kg)
B
MS (100mg/kg) Pretreatment
Number of jumps/15min
100
75
50
25
0
***
***
#
##
Saline
0
20
200
400
Unformulated Curcumin (mg/kg)

Fig. 2

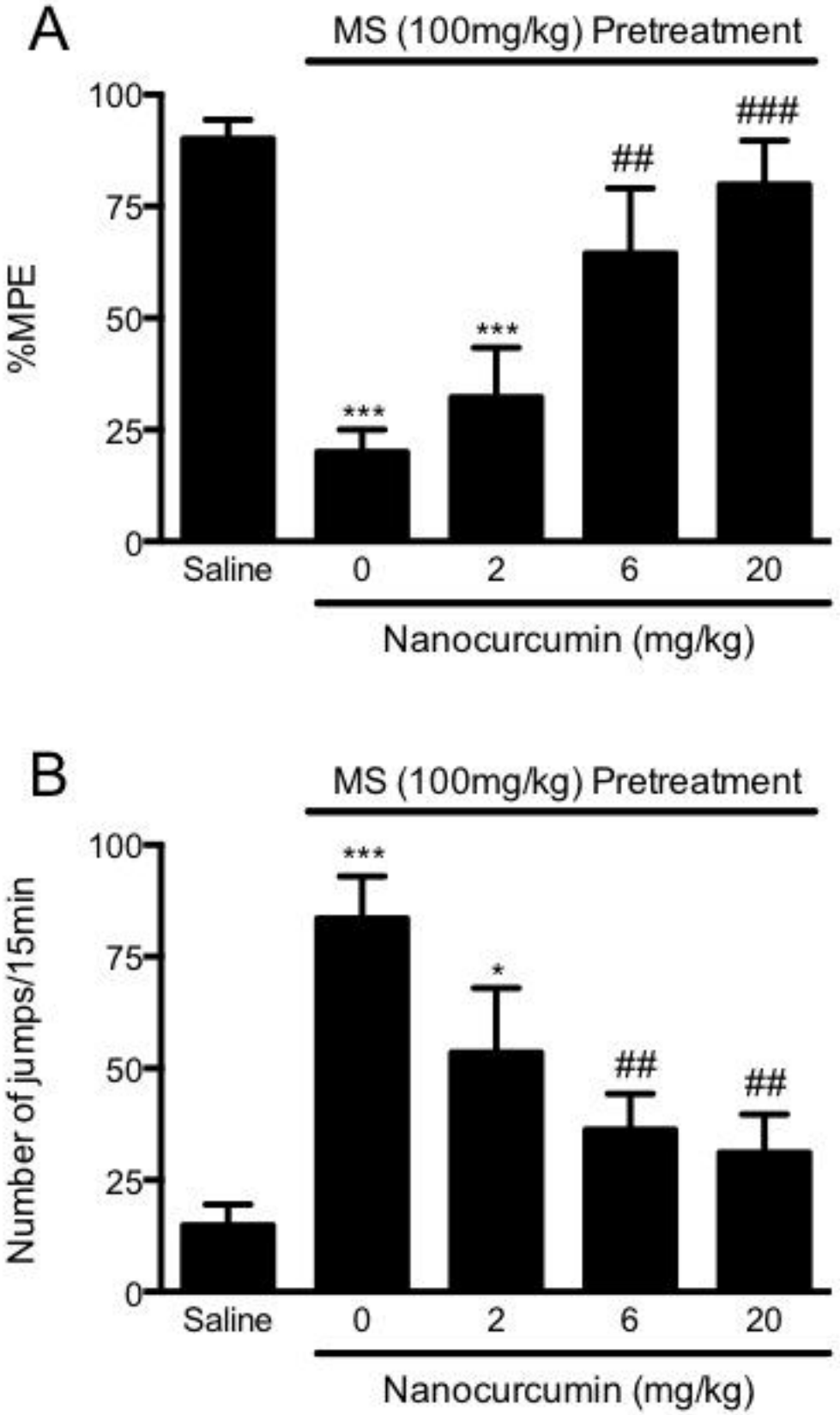
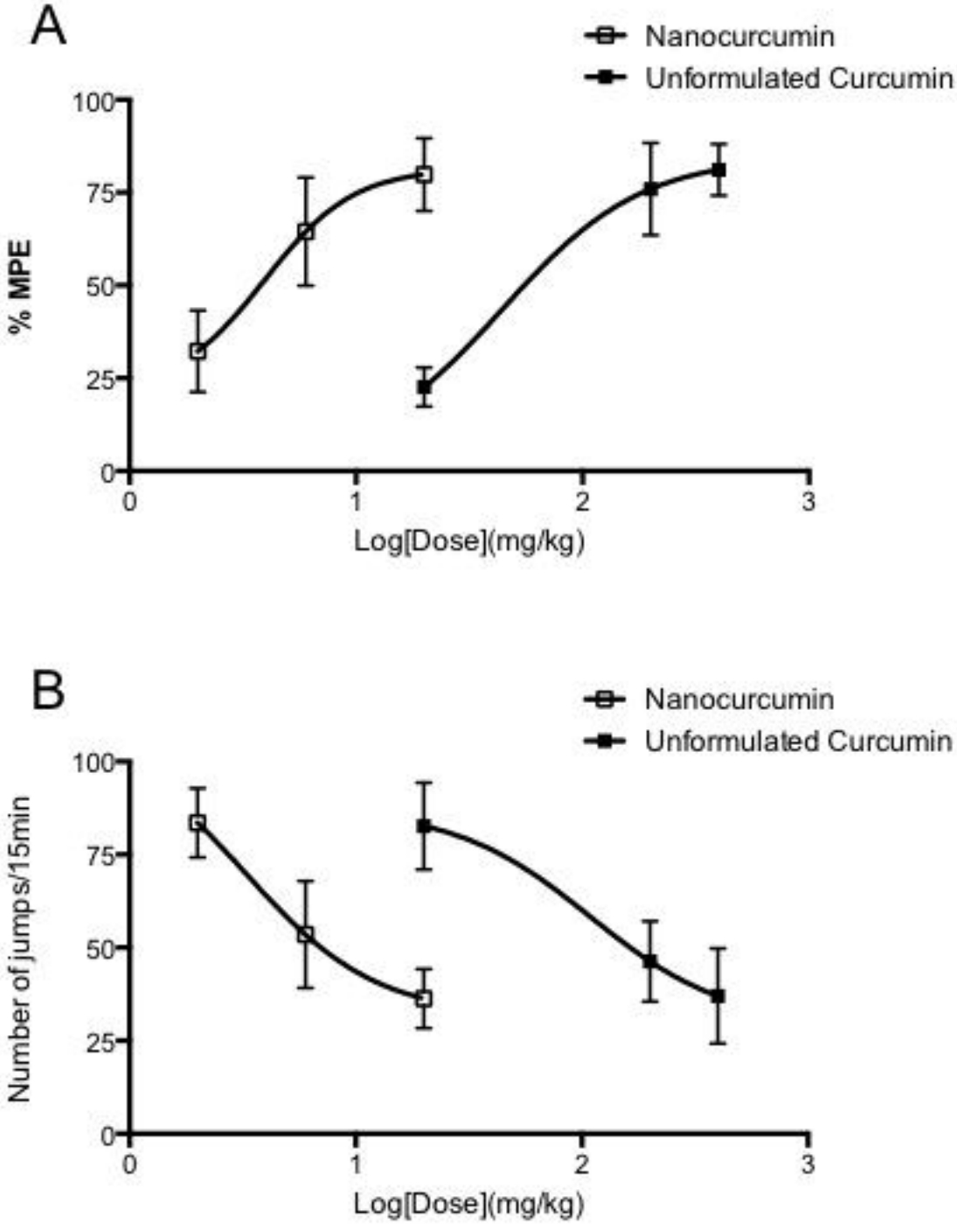

A
MS (100mg/kg) Pretreatment
%MPE
100
75
50
25
0
***
***
##
###
Saline
0
2
6
20
Nanocurcumin (mg/kg)
B
MS (100mg/kg) Pretreatment
Number of jumps/15min
100
75
50
25
0
***
*
##
##
Saline
0
2
6
20
Nanocurcumin (mg/kg)

Fig. 3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Nanocurcumin
Unformulated Curcumin
% MPE
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75
50
25
0
0
1
2
3
Log[Dose](mg/kg)
B
Nanocurcumin
Unformulated Curcumin
Number of jumps/15min
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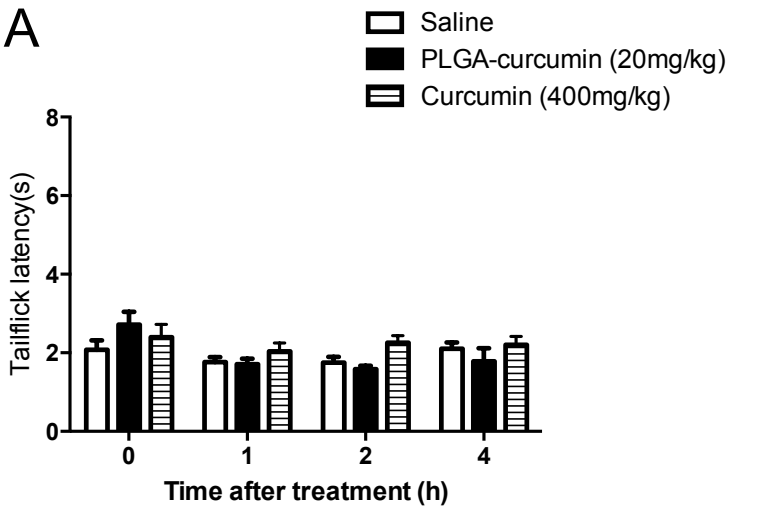
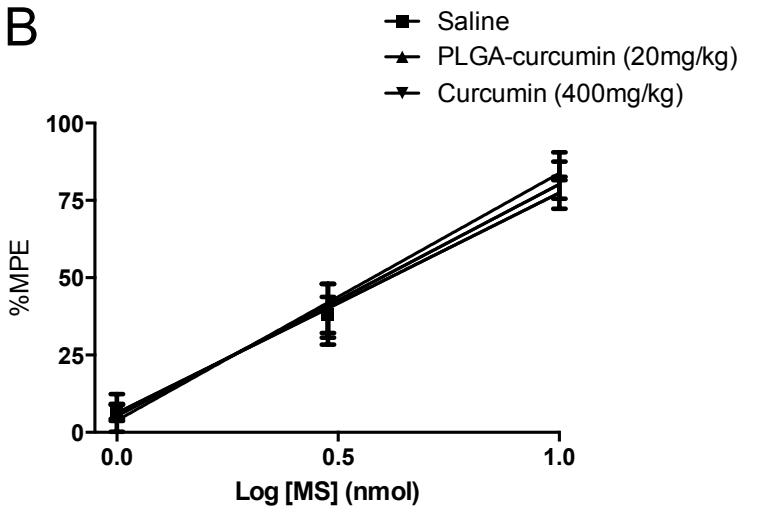
75
50
25
0
0
1
2
3
Log[Dose](mg/kg)

Fig. 4


A
Saline
PLGA-curcumin (20mg/kg)
Curcumin (400mg/kg)
Tailflick latency(s)
8
6
4
2
0
0
1
2
4
Time after treatment (h)


B
Saline
PLGA-curcumin (20mg/kg)
Curcumin (400mg/kg)
%MPE
100
75
50
25
0
0.0
0.5
1.0
Log [MS] (nmol)

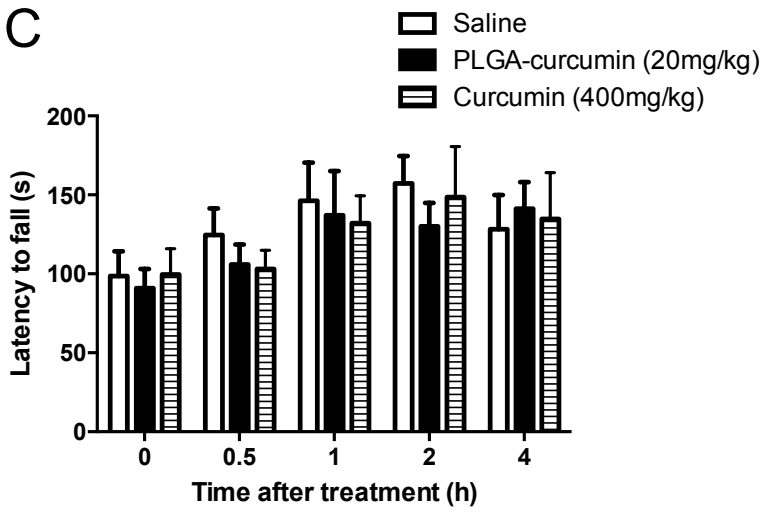

C
Saline
PLGA-curcumin (20mg/kg)
Curcumin (400mg/kg)
Latency to fall (s)
200
150
100
50
0
0
0.5
1
2
4
Time after treatment (h)

Fig. 5

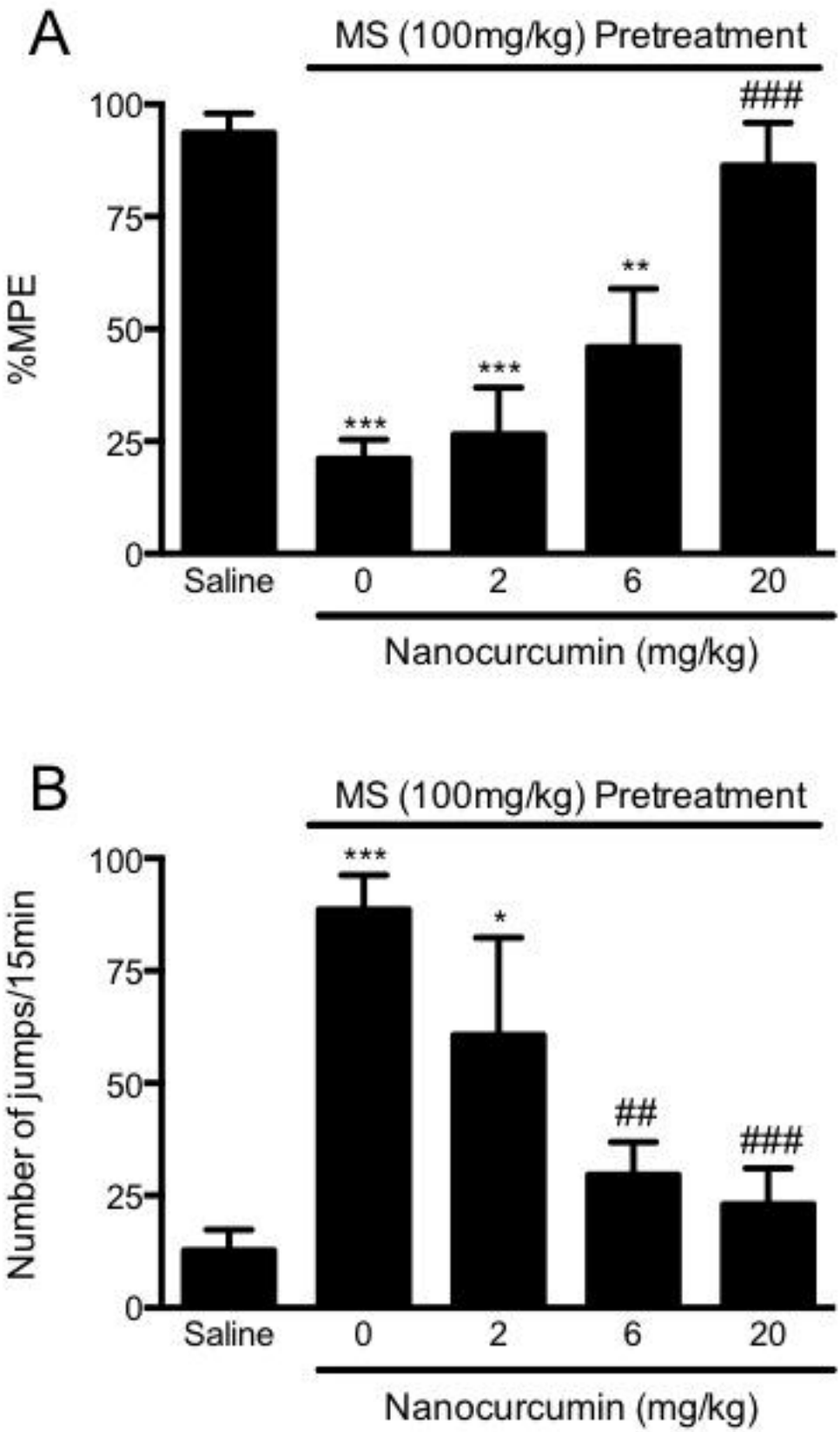

A
MS (100mg/kg) Pretreatment
%MPE
100
75
50
25
0
***
***
**
###
Saline
0
2
6
20
Nanocurcumin (mg/kg)
B
MS (100mg/kg) Pretreatment
Number of jumps/15min
100
75
50
25
0
***
*
##
###
Saline
0
2
6
20
Nanocurcumin (mg/kg)

Fig. 6

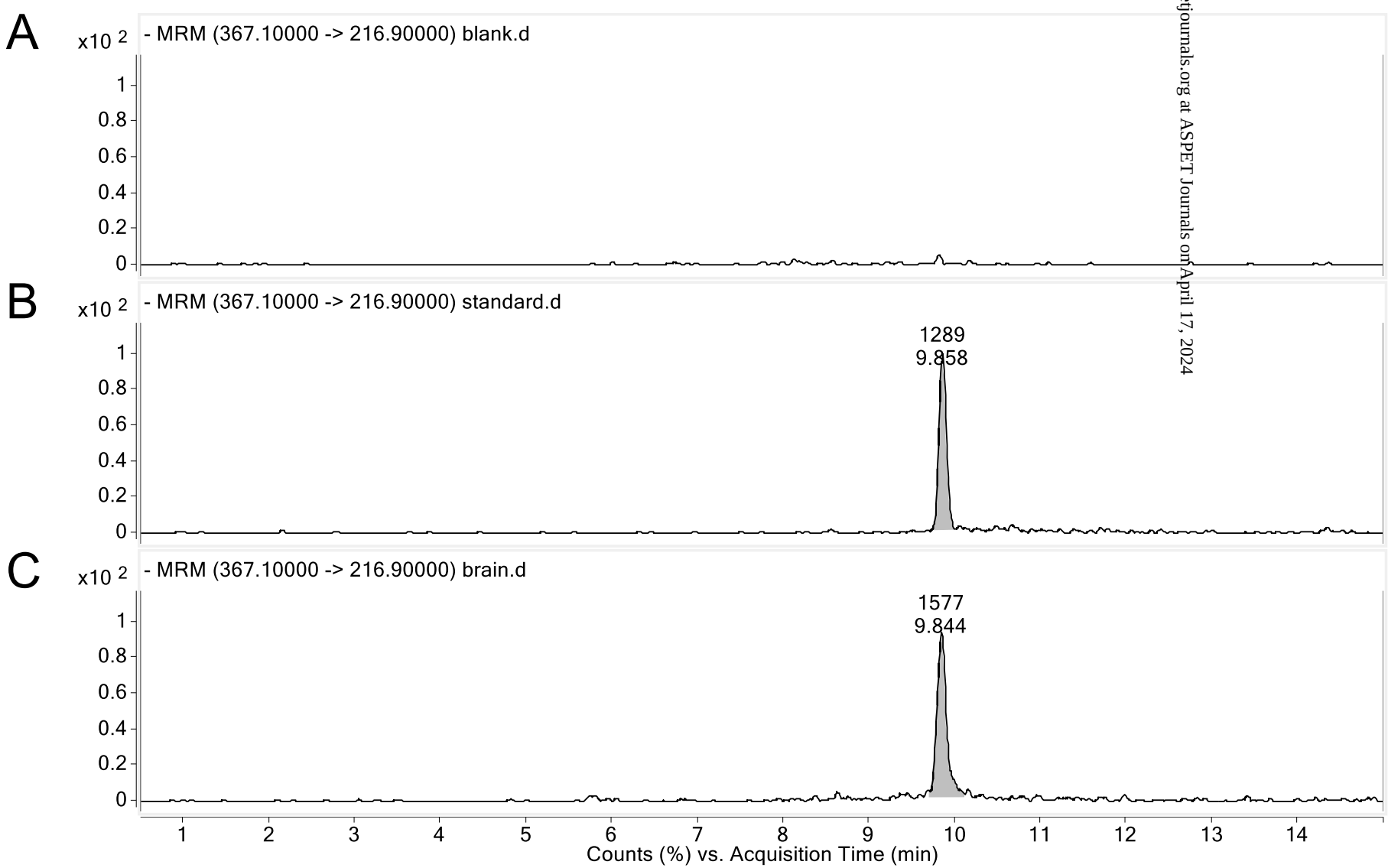
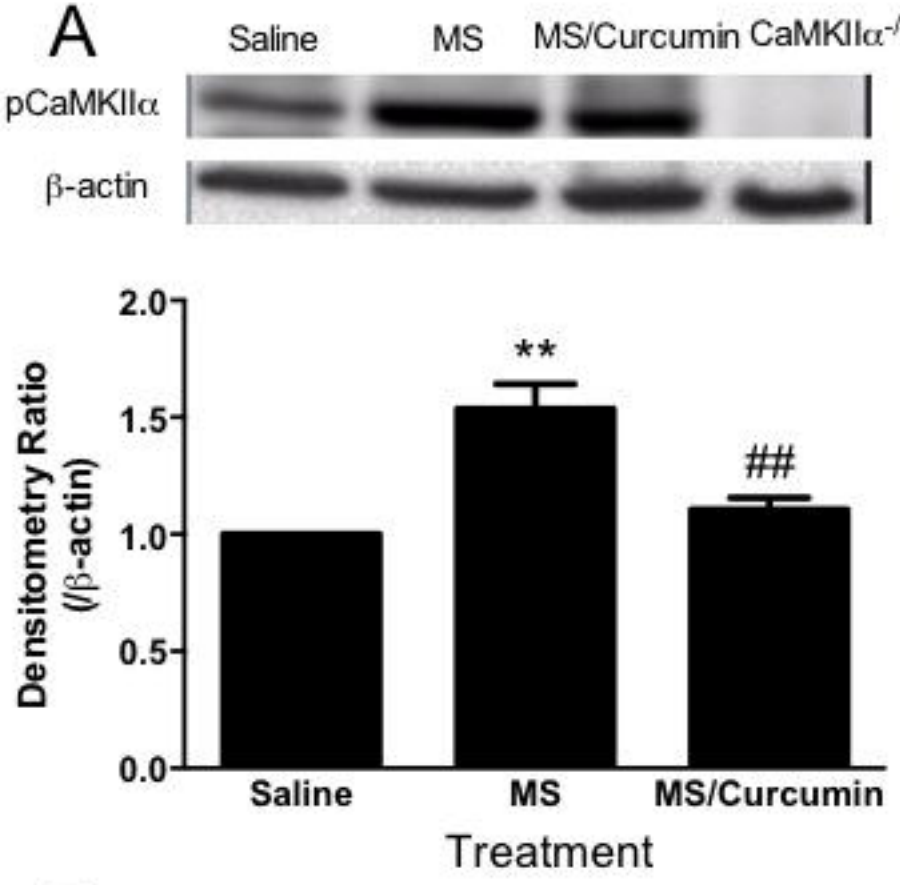


Fig. 7


A
Saline
MS
MS/Curcumin
CaMKIIα-/-
pCaMKIIα
β-actin
Densitometry Ratio (/β-actin)
2.0
1.5
1.0
0.5
0.0
**
##
Saline
MS
MS/Curcumin
Treatment

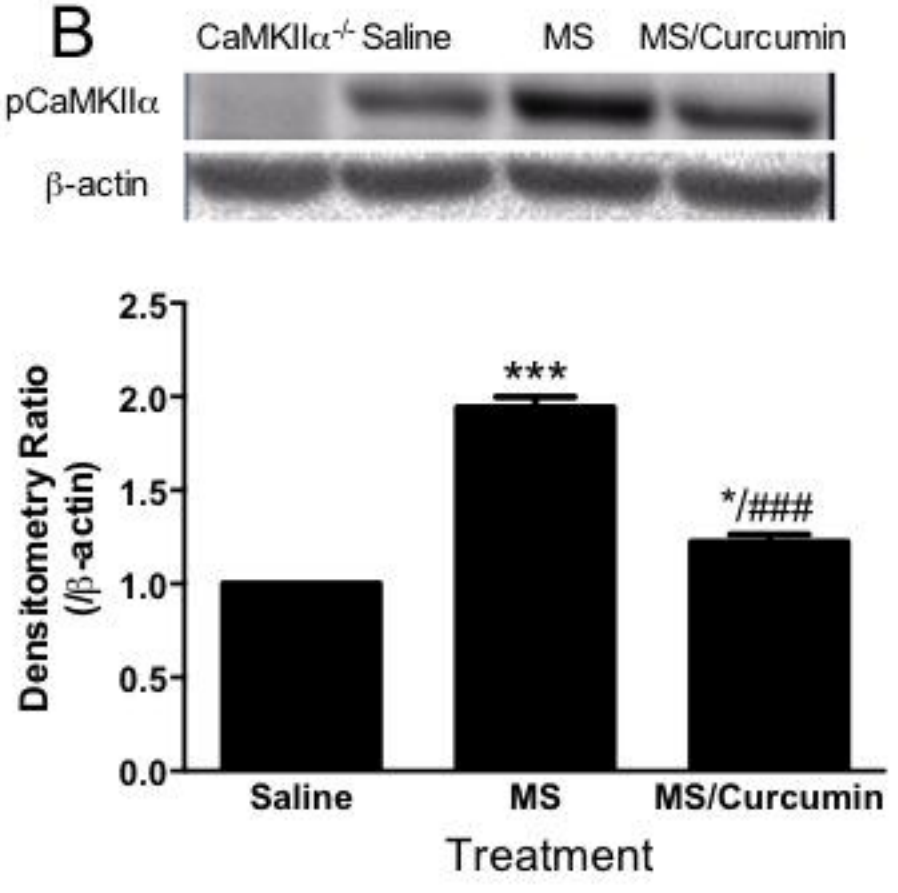
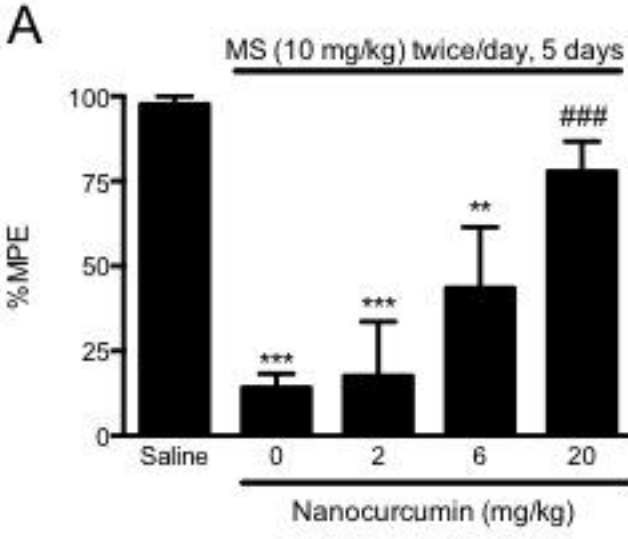

B
CaMKIIα-/-
Saline
MS
MS/Curcumin
pCaMKIIα
β-actin
Densitometry Ratio (/β-actin)
2.5
2.0
1.5
1.0
0.5
0.0
***
*/###
Saline
MS
MS/Curcumin
Treatment

Fig. 8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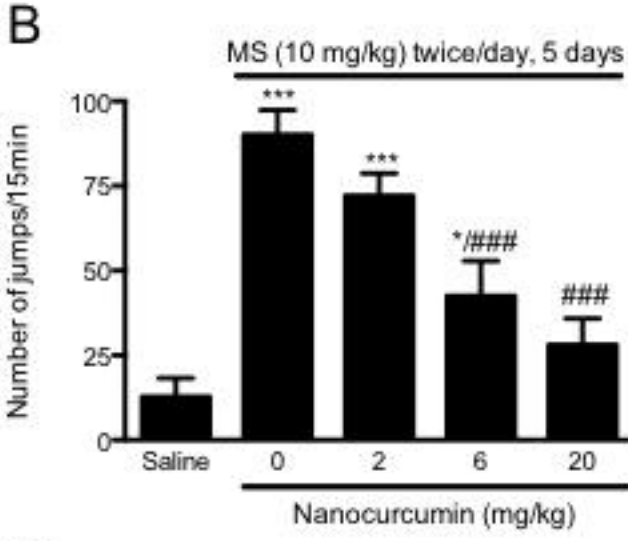
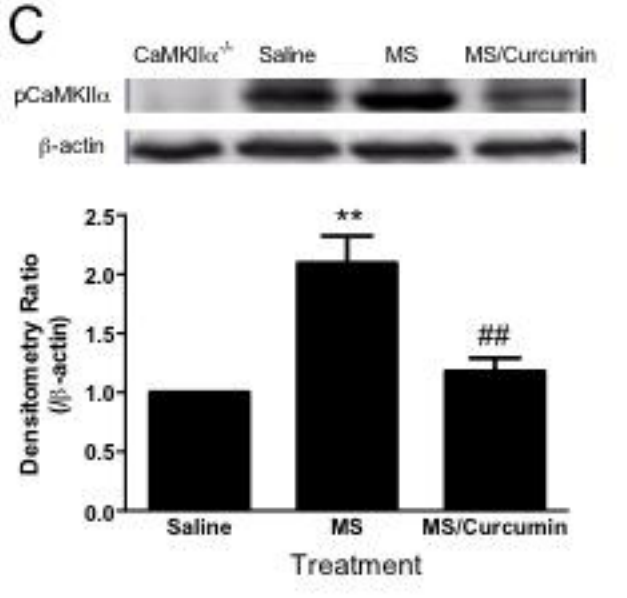
MS (10 mg/kg) twice/day, 5 days
%MPE
100
75
50
25
0
***
***
**
###
Saline
0
2
6
20
Nanocurcumin (mg/kg)


B
MS (10 mg/kg) twice/day, 5 days
Number of jumps/15min
100
75
50
25
0
***
***
*/###
###
Saline
0
2
6
20
Nanocurcumin (mg/kg)


C
CaMKIIα-/-
Saline
MS
MS/Curcumin
pCaMKIIα
β-actin
Densitometry Ratio (/β-actin)
2.5
2.0
1.5
1.0
0.5
0.0
**
##
Saline
MS
MS/Curcumin
Treatment