Role of CaMKIIα Leading to Inflammatory and Neuropathic Pain

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THESIS

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LIST OF ABBREVIATIONS

AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid
ASIC	acid-sensitive ion channels
CaMKII	Calcium/calmodulin-dependent Protein Kinase II
CCI	Chronic constriction injury
CFA	Complete Freund's adjuvant
CNS	Central Nervous System
COX	Cyclooxygenase
DRG	Dorsal Root Ganglion
EDTA	Ethylenediaminetetraacetic Acid
GPCR	G protein-coupled receptors
IASP	International Association for the Study of Pain
KN92	2-[N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine
KN93	[2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine
LTP	Long-term potentiation
MS	Morphine Sulfate
NMDA	N-methyl-D-aspartate
OIH	opioid-induced hyperalgesia
PBS	Phosphate Buffered Saline
PKC	Protein Kinase C

LIST OF ABBREVIATIONS (continued)

RNAi	RNA Interference
SDS	Sodium Dodecyl Sulfate
siRNA	Small Interfering RNA
SNL	Spinal nerve ligation
TFP	Trifluoperazine
TRP	Transient receptor potential channels
TTX	Tetrodotoxin

I. INTRODUCTION

1. Background

Pain, as defined by the International Association for the Study of Pain (IASP, 1994), is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. Although being unpleasant, pain is essential to an organism's survival from potential hazardous situations (Ji and Strichartz, 2004). A simple example is diabetes caused impairment of pain sensation and in those patients, the risk of severe tissue damage is greatly increased (Brookoff, 2000).

Pain can be further classified as acute and chronic pain. As an alarm system, acute pain is triggered by noxious stimuli acting on a specialized high-threshold sensory system (Scholz and Woolf, 2002). Pain signals are generated by peripheral afferent sensory neurons called nociceptors (Coutaux et al., 2005). With free nerve ending located at peripheral tissues, nociceptors receive noxious stimuli and transduce signals into action potentials. Nerve fibers Aδ and C pass the information to second order neurons in the dorsal horn of the spinal cord, where the input is conveyed and relayed to higher centers via ascending pathways: the spinothalamic and spinoparabrachial pathways (Coutaux et al., 2005).

After tissue injury or nerve damage, alterations of the pain pathway may display enhanced sensitivity. For example, low-threshold sensory fibers, normally only respond to innocuous sensation, can begin to produce pain, referred to as allodynia, or the sensitization of nociceptive neurons can lead to an aggregated response to

noxious stimuli, termed hyperalgesia (Coutaux et al., 2005). Associated with prolonged tissue damage or injuries to the nervous system, pain signals keep firing for weeks, months, even years, resulting in chronic pain (Dray et al., 1994). Common chronic pain complaints include headache, cancer pain, low back pain, arthritis pain, etc. In the United States, chronic pain is currently one of the most serious health problems and causes long-term disability upwards of 50 million people (Brookoff, 2000).

Based on the initiation conditions and the anticipated underlying mechanisms, persistent pain has been further divided into inflammatory or neuropathic pain (Scholz and Woolf, 2002; Xu and Yaksh, 2011). A variety of events contributed to sensation of inflammatory pain, including penetration wounds, extreme cold, arthritis, infections, cancer cell growth, and autoimmune conditions, etc. At the peripheral terminal, damaged tissue release a large range of inflammatory molecules including cycoloxygenase activates prostaglandin and leukotrienes resulting from cellular breakdown, neuropeptides secreted by primary afferent neuron, cytokines, neurotropins, serotonin, and histamine produced by activated immune cells, as well as extracellular proteases and protons (Dray, 1995; Scholz and Woolf, 2002). Referred to as “inflammatory soup”, some of these substances are neuroactive, which directly stimulate chemosensitive nociceptors through G protein-coupled receptors (GPCR), transient receptor potential channels (TRP), and acid-sensitive ion channels (ASIC), located on the afferent terminals (Coutaux et al., 2005; Xu and Yaksh, 2011). While other chemical mediators act via peripheral and central sensitization, which enhance the excitability of nerve fibers to low-intensity stimuli, thus playing a major role in the

progression to inflammatory pain (Coutaux et al., 2005). In the dorsal horn of spinal cord, central sensitization happens quickly via activity-dependent receptors/ion channels phosphorylation or trafficking (Scholz and Woolf, 2002). For example, tissue injuries- activated protein kinase C (PKC) enhance the N-methyl-D-aspartate (NMDA) receptor by removing Mg^{2+} blockage (Chen and Huang, 1992); painful stimuli increased trafficking of 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) subunits to the plasma membrane of spinal cord neurons (Galan et al., 2004). On the other hand, the response can also be sustained by transcriptional changes of proteins or structural alterations in neurons, including the expression of receptors, neurotransmitters, ion channels, cell loss, as well as synaptic reorganizations (Dray et al., 1994; Scholz and Woolf, 2002).

As early as 1892, Mitchell has provided a systematic description of posttraumatic neuropathic pain (Mitchell, 1872). Defined as abnormal pain sensation, neuropathic pain is caused by lesions to or dysfunction in the peripheral or central nervous system (Scholz and Woolf, 2002; Ji and Strichartz, 2004; Ueda, 2006). Diverse disease conditions, such as spinal cord injury, stroke, degenerative neurological diseases, diabetes and cancer, can result in neuropathic pain (Scholz and Woolf, 2002; Woolf, 2004). As the onset of neuropathic pain may be consistent after healing is a complete, neuropathic pain present as a most difficult task for diagnosis and treatment is also a challenge with only 40-60% of patients achieving partial relief (Ueda, 2006; Dworkin et al., 2007). Similar as inflammatory pain, peripheral and central sensitization contribute to the initiation and procession of neuropathic pain.

These include changes in injured and neighboring non-injured sensory neurons, as well as transynaptic changes at multiple levels of the CNS (Woolf and Mannion, 1999; Woolf, 2004). Following a nerve injury, there is an increase level of spontaneous firing in primary afferent neurons, which rarely happens in normal condition. This has been termed “ectopic discharge” and is possibly caused by the chemically cross-excitation/ cross-depolarization by neighboring neurons. As a result, both injured and their uninjured neighbors develop ectopic activity and contribute to the initiating and maintaining of pain sensation (Wall et al., 1974; Amir and Devor, 1996; Gold, 2000; Bridges et al., 2001). In addition, the alteration in ion channels expression also increases the excitability of the neurons, possibly lead to an increase in firing level and frequency, including up-regulation of type III tetrodotoxin (TTX)-sensitive Na^+ channel and down-regulation of α-SNS (PN3), NaN (SNS2) subunits of TTX-resistant Na^+ channels, as well as loss of certain N-type Ca^{2+} channels (Waxman et al., 1994; Dib-Hajj et al., 1996; Bridges et al., 2001; Altier et al., 2006; Altier et al., 2007). Increased long-lasting discharge of peripheral neurons also causes plastic changes in the spinal cord and brain, leading to central sensitization. In a very similar fashion to persistent inflammatory pain, central sensitization has been proposed as a key mechanism underlying chronic neuropathic pain by: 1) amplifying processing of input from injured peripheral nerve; 2) Increasing response for sub-threshold inputs or input from uninjured tissue; 3) Expanding the receptive area of the spinal cord (Woolf and Salter, 2000; Schaible and Richter, 2004; Basbaum et al., 2009). Central sensitization could be activity-dependent (due to phosphorylation of ion channels or receptors and

activation of intracellular kinases) or transcription-dependent (due to enhanced gene expression and removal of repressor), pertinent to relative immediate or long-lasting changes in the function of dorsal horn neuron (Scholz and Woolf, 2002). Accumulative evidence has revealed several receptor/transmitter systems involved in the mechanism. Glutamate is the main transmitter in pain processing, which activates NMDA and non-NMDA receptors (Fundytus, 2001). In normal condition, acting of glutamate on non-NMDA receptor causes influx of Na^+, resulting in depolarization of neuron membrane. Persistent membrane depolarization further activates NMDA receptors and provides an opening for Ca^{2+}. Then large amount of Ca^{2+} actives second-messenger cascades and triggers downstream signaling, resulting in neural hyper-excitation (Woolf and Thompson, 1991; Xu et al., 1992; Felsby et al., 1996; Woolf, 2004).

Since current knowledge regarding central sensitization is incomplete, the study of mechanism of pain is in great need. Here we focus on Ca^{2+}/calmodulin dependent protein kinase II (CaMKII). As one of the must aboundent proteins, it counts 1% of total protein in forebrain and 2% in hippocampus (Erondu and Kennedy, 1985). CaMKII is serine/ threonine-specific protein kinase, primarily regulated by the Ca^{2+}/calmodulin complex. So far four isoforms of CaMKII have been dicovered (α, β, γ and δ), within which, CaMKIIα and CaMKIIβ are the two major isoforms (Petrenko et al., 2003). Previous studies have revealed the function of CaMKII in modulating neurotransmitter receptors, ion channels and neurotransmitter synthesis. More importantly, it plays an important role in central sensitization and long term potentiation,

which contribute to chronic pain and memory (Leonard et al., 1999; Strack et al., 2000; Petrenko et al., 2003; Schmitt et al., 2005). Pharmacologically study of the role of CaMKII in pain mechanism may open the door for novel analgesics effective drugs.

2. Purpose of Study

In this study, we employed three animal models to investigate the role of CaMKII in the initiation and maintenance of pain. Furthermore, the effect of CaMKII inhibition on complete Freund's adjuvant (CFA)-induced inflammatory pain, spinal nerve ligation (SNL)-induced neuropathic pain, as well as opioid-induced hyperalgesia (OIH) are were evaluated.

Conflicting data exist in the literature for the role of CaMKII in pain processing. In the present study, we first hypothesis that CaMKII is required for the generation and maintenance of inflammatory pain in a rodent model induced by CFA. Intraplantarly injected CFA was found to induce spinal activity of CaMKII (phosphorylated CaMKII), which was blocked by KN93 [[2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine], a CaMKII inhibitor. Pretreatment with KN93 (i.t.) dose-dependently prevented the development of CFA induced thermal hyperalgesia and mechanical allodynia. Acute treatment with KN93 (i.t.) also dose-dependently reversed CFA induced thermal hyperalgesia and mechanical allodynia. Furthermore, our previous studies identified trifluoperazine, a clinically used antipsychotic drug, to be a potent CaMKII inhibitor. Inhibition of CaMKII activity by trifluoperazine was confirmed in the study. In addition, trifluoperazine (i.p.)

dose-dependently reversed CFA-induced mechanical allodynia and thermal hyperalgesia. The drug was also effectively when given orally. Our findings support a critical role of CaMKII in inflammatory pain.

Based on the fact that inflammatory and neuropathic pain often share common mechanism, next we evaluated the role of CaMKII in a rodent model of SNL-induced experimental mononeuropathy. Tight ligation of L5 and L6 nerves (SNL) successfully developed mechanical and thermal hypersensitivity in mice. While acute treatment with KN93 (i.t.) dose-dependently reversed SNL-induced thermal hyperalgesia and mechanical allodynia. We further examine the pharmacological action of the antipsychotic trifluoperazine, in these assays. Trifluoperazine also (i.p. or p.o.) dose-dependently reversed SNL-induced mechanical allodynia, thermal hyperalgesia, and CaMKII activation. In conclusion, our findings support a critical role of CaMKII in neuropathic pain.

Opioids have been widely used to pain management, while repeated administration of opioids not only leads to tolerance and dependence, but also results in nociceptive enhancement called opioid-induced hyperalgesia (OIH). Nociceptive mediators involved in OIH generation remain poorly understood. In the present study, we tested the hypothesis that CaMKIIα is critical for OIH. Opioid-induced hyperalgesia was produced by repeated morphine administration or pellet implantation in mice. Correlating with the development of tactile allodynia and thermal hyperalgesia, spinal CaMKIIα activity was significantly increased in OIH. KN93 dose- and time-dependently reversed OIH and CaMKII activation without impairing locomotor

coordination. To elucidate the specific CaMKII isoform involved, we targeted CaMKIIα by employing small interfering RNA and demonstrated that knockdown of spinal CaMKIIα attenuated OIH. Furthermore, morphine failed to induce OIH in CaMKIIαT286A point mutant mice, although wild-type littermate mice developed robust OIH after repeated treatments with morphine. These data implicate, for the first time, an essential role of CaMKIIα as a cellular mechanism leading to and maintaining opioid-induced hyperalgesia.

II. REVESAL OF CHRONICA INFLAMMATORY PAIN BY ACUTE INHIBITION OF Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II

1. Introduction

Peripheral nerve injury or tissue inflammation often leads to pathologic persistent pain that is presented with spontaneous pain, hyperalgesia, and allodynia, which may be mediated through both peripheral and central mechanisms (Dubner and Ruda, 1992; McMahon et al., 1993; Willis, 2001). Central sensitization resulting from neuronal plasticity in the dorsal horn of the spinal cord is a pivotal mechanism underling the development of hyperalgesia and allodynia (Woolf, 1983; Coderre et al., 1993). However, the specific pathways leading to central sensitization remain unclear. A number of receptors, including the N-methyl-D-aspartate (NMDA) receptors, have been found to contribute to the development of central sensitization (Woolf and Thompson, 1991; Xu et al., 1992). Activation of these receptors initiates cascades of intracellular signaling events involving Ca^{2+} and various protein kinases (Lin et al., 1996; Malmberg et al., 1997). For example, activation of the NMDA receptors causes Ca^{2+} influx, leading to changes in neuronal plasticity (Womack et al., 1988). Considerable evidence has demonstrated that Ca^{2+}-mediated cell signaling is important in nociception (Saegusa et al., 2001; Kim et al., 2003). One action of Ca^{2+} is through its activation of calmodulin, which in turn triggers the activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). CaMKII is expressed in the superficial laminae of the dorsal horn of the spinal cord and in the small to medium

diameter primary sensory neurons in dorsal root ganglia, where nociceptive signals are transmitted and processed (Bruggemann et al., 2000; Carlton, 2002). The function of CaMKII in pain is somewhat mixed. Fang et al. (2002) reported that CaMKII activity [determined by phosphorylated CaMKII (pCaMKII)] was significantly increased in the spinal cord within minutes after an intradermal injection of capsaicin (Fang et al., 2002). The second phase of formalin-induced paw-licking behavior was significantly reduced in CaMKIIα (T286A) mutant mice that are unable to be autophosphorylated and activated; however, mutant and wildtype mice similarly showed decreased thermal and mechanical thresholds induced by complete Freund's adjuvant (CFA) or formalin (Zeitz et al., 2004). On the contrary, it was reported that KN93, a CaMKII inhibitor, was capable of preventing the development of thermal hyperalgesia and mechanical allodynia following chronic constriction injury (CCI) (Dai et al., 2005) or inferior alveolar nerve transaction (Ogawa et al., 2005). KN93 selectively and directly binds to the CaM-binding site of CaMKII, preventing the activation of CaMKII (Sumi et al., 1991). However, KN93 was found to be ineffective when given 7-day postinjury (0.25 μg/μl/h i.t. via an Alzet pump for 7 days) to reverse established hyperalgesia and allodynia in CCI model (Dai et al., 2005). It is not known whether the lack of acute effects by KN93 was dose-related. Previously, we found that different doses of CaMKII inhibitors were required to disrupt opioid tolerance depending on the degree of opioid tolerance (Tang et al., 2006a).

In this study, we tested the hypothesis that sufficient CaMKII inhibition is capable of acutely reversing already established inflammatory pain. Acute actions of

CaMKII inhibitors in CFA-induced inflammatory pain were examined. Investigating an acute action of CaMKII inhibition in chronic pain is critical not only for understanding the mechanisms but more importantly for designing useful drug therapies of chronic inflammatory pain. Most patients seek medical treatment of chronic pain after the initial nerve or tissue injuries.

2. Materials and Methods

2.1 Materials

CFA (0.5 mg/ml Mycobacterium tuberculosis (H 37RA, ATCC 25177) suspended in an oil/saline (1:1) emulsion and trifluoperazine were purchased from Sigma (St. Louis, MO). KN93 and KN92 were from Calbiochem (San Diego, CA).

2.2 Animals

Male ICR (Institute of Cancer Research) mice (25 ± 5 g; Harlan Laboratories, Indianapolis, IN) were kept in a vivarium with a 12 - h alternating light/dark cycle and food and water available ad libitum. All experiments were performed during the light cycle. Mice were randomly divided into experimental groups according to a computer-generated randomization list. All procedures were performed in accordance with the policies and recommendations of the International Association for the Study of Pain and the Institute of Laboratory Animal Resources (1996) and approved by the University of Illinois Institutional Animal Care and Use Committee.

2.3 Methods

2.3.1 CFA-Induced Inflammatory Pain

Unilateral inflammation was induced by injecting 20 μl of CFA into mice dorsal surface of the left hindpaw (i.pl.) as described previously (Tang et al., 2007a). Control mice received 20 μl of saline. Mice were tested for thermal hyperalgesia and mechanical allodynia before and 24 and 72 h after CFA injection.

2.3.2 Drug Administration

Intrathecal injection (i.t.) was given in a volume of 5 μl by percutaneous puncture through an intervertebral space at the level of the 5th or 6th lumbar vertebra, as described previously (Hylden and Wilcox, 1980; Wang et al., 2001). Success of the i.t. injection was verified by a lateral tail-flick. The pretreatment group was given KN93 (5–30 nmol i.t.) 30 min before CFA injection. For acute reversal experiments, mice were administered with KN93 (15–45 nmol i.t.) or KN92 (45 nmol i.t.) 2 h before pain testing on days 1 and 3 post-CFA injection. Trifluoperazine was administered i.p. (0.1 - 0.5 mg/kg) or via gastric gavage (1 mg/kg) 2 h before pain testing on day 1 post-CFA injection. In these studies, control mice received an equal volume of saline.

2.3.3 Thermal Hyperalgesia

The paw-withdrawal latencies to heat stimuli were measured using a plantar tester (model 7372; Ugo Basile, Comerio, Italy) as described previously (Hargreaves et al., 1988; Wang et al., 2001; Tang et al., 2007a). Mice were placed in a clear plastic

cage on a glass floor. After a 30-min period of habituation, paw-withdrawal latencies to radiant heat stimulation were measured. The radiant heat source was focused on the middle portion of the plantar surface of the left hindpaw and was automatically ceased when a paw-withdrawal occurred. A cut-off time of 20 s was applied to prevent tissue damage.

2.3.4 Mechanical Allodynia

Mechanical allodynia was measured using calibrated von Frey filaments (Stoelting, Wood Dale, IL) as described previously (Chaplan et al., 1994; Tang et al., 2007a). In brief, mice were placed into individual Plexiglas containers with a wire mesh floor and allowed to acclimate for 30 min before testing. Each von Frey filament was applied perpendicularly to the midplantar surface for 5 s or until a withdrawal response had occurred. The up-down paradigm was used to determine 50% probability of paw withdrawal threshold (Dixon, 1980; Chaplan et al., 1994; Wang et al., 2001).

2.3.5 CaMKII Activity Assay

The CaMKII activity was measured based on a previously published method (Ocorr and Schulman, 1991). Mouse brain tissues containing CaMKII were extracted with the extraction buffer containing 20 mM Tris-HCl, pH 8.0, 1 Mm EGTA, 10 mM sodium pyrophosphate, 25 mM benzamidine, 20 mM soybean trypsin inhibitor, 10 mM aprotinin, 5 mM leupeptin, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. After homogenization and centrifugation (45,000g, 60 min), 2.5 μl supernatant (10 μg

of protein) was added into 22.5 µl of reaction mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.5 mM dithiothreitol, 5 mM β-glycerol phosphate, 0.2 mM sodium orthovanadate, 1 µM calmodulin, 1 mM $CaCl_2$, 0.02 mg/ml BSA, 100 µM ATP, 0.5 µCi of [γ-^{32}P] ATP (3000 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA) and 0.1 mM autocamtide-2 at 30°C for 5min. To determine the Ca^{2+}/calmodulin-independent protein kinase activity of CaMKII, reactions were carried out under the same condition except for the presence of 1 mM EGTA and omission of $CaCl_2$ and calmodulin. Reactions were stopped by spotting onto P81 phosphocellulose paper and immediately washed with 75 mM H_3PO_4 three times. Incorporation of ^{32}P was quantified by liquid scintillation counting of the P81 paper. Percentage of inhibition of CaMKII activity was calculated (Ocorr and Schulman, 1991).

2.3.6 Western Blotting Analysis

Immediately after the behavior test, lumbar sections of spinal cord were quickly dissected from euthanized mice and frozen on dry ice for Western blotting analysis as described previously (Tang et al., 2006a). In brief, tissues were homogenized using a glass homogenizer in 200 µl of radioimmunoprecipitation assay buffer [1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 5 mM EDTA in phosphate buffered saline, pH 7.4] in the presence of phosphatase inhibitors (10 mM sodium fluoride, 10 mM sodium pyrophosphate, and 1 µM okadaic acid) and protease inhibitors (0.05 mg/ml bestatin, 0.05 mg/ml leupeptin, 0.05 mg/ml pepstatin, and 0.1 mg/ml phenylmethylsulfonyl fluoride). The homogenates were

incubated on a rotator at 4°C for 2 h, and the soluble fraction was separated by centrifugation (45,000g, 60 min). Protein content in the supernatant was determined by a modified Bradford method (Pierce Biotechnology, Rockford, IL). Samples (60 μg of protein) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene difluoride membrane. The membrane was preblocked in 5% nonfat milk in 20 mM Tris-buffered saline, pH 7.6, with 0.1% Tween 20 and probed with a rabbit anti-(T286) pCaMKII antibody (1:1000; Promega, Madison, WI). The membrane was then washed and incubated with a donkey anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody (1:1000; Amersham, Piscataway, NJ), washed, and developed using an enhanced chemiluminescence detection system (ECL; Amersham). The membrane was then stripped and reprobed with a mouse anti-β-actin antibody (1: 10,000; Sigma) followed by another incubation with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:10,000; Amersham) and developed as above. ECL signals were captured by a ChemiDoc imaging system and analyzed using the Quantity One program (Bio-Rad, Hercules, CA). Ratios of the optical densities of pCaMKII to those of β-actin were calculated for each sample.

2.3.7 Statistical Analysis

Comparisons between groups were analyzed using a two-way repeated measure ANOVA followed by post hoc analyses using Dunnett's t test. Statistical significance was established at the 95% confidence limit.

3. Results

3.1 Effect of CaMKII Inhibition on Preventing CFA-Induced Hyperalgesia

Thermal hyperalgesia and mechanical allodynia are known to develop after an intraplantar CFA injection. CFA-treated mice demonstrated significantly reduced withdrawal latencies to radiant heat and decreased withdrawal thresholds to von Frey filaments within 24 h (Fig. 1). We examined whether blocking the activation of CaMKII could prevent the development of pain behavior induced by CFA. Intrathecal administration of KN93 (30 nmol), a CaMKII inhibitor (Niki et al., 1993), significantly blocked the development of mechanical allodynia (Fig. 1A) and thermal hyperalgesia (Fig. 1B) ($p < 0.001$ compared with the CFA group, $n = 8$). We further examined the CaMKII activity (pCaMKII) after CFA injection by the Western blotting analysis and found that pCaMKII was significantly upregulated by CFA [Fig. 2, lane 2, $p < 0.05$ compared with the control group (lane 1), $n = 4$]. Pretreatment with KN93 (30 nmol i.t.) prevented the CFA-induced increase of pCaMKII (Fig. 2, lane 6, $p < 0.05$ compared with the CFA group, $n = 4$). Pretreatment with CFA or CFA + KN93 (30 nmol) did not seem to significantly alter CaMKII protein expression, although there is a general trend of slight up-regulation (Fig.3). KN93 (15 nmol) was partially effective in blocking the CFA-induced mechanical allodynia but not thermal hyperalgesia (Fig. 1). At an even lower dose, KN93 (5 nmol) was not active in either assay. ED50 values for KN93 were estimated to be 15.9 (blocking allodynia) and 19.4 nmol (blocking hyperalgesia).

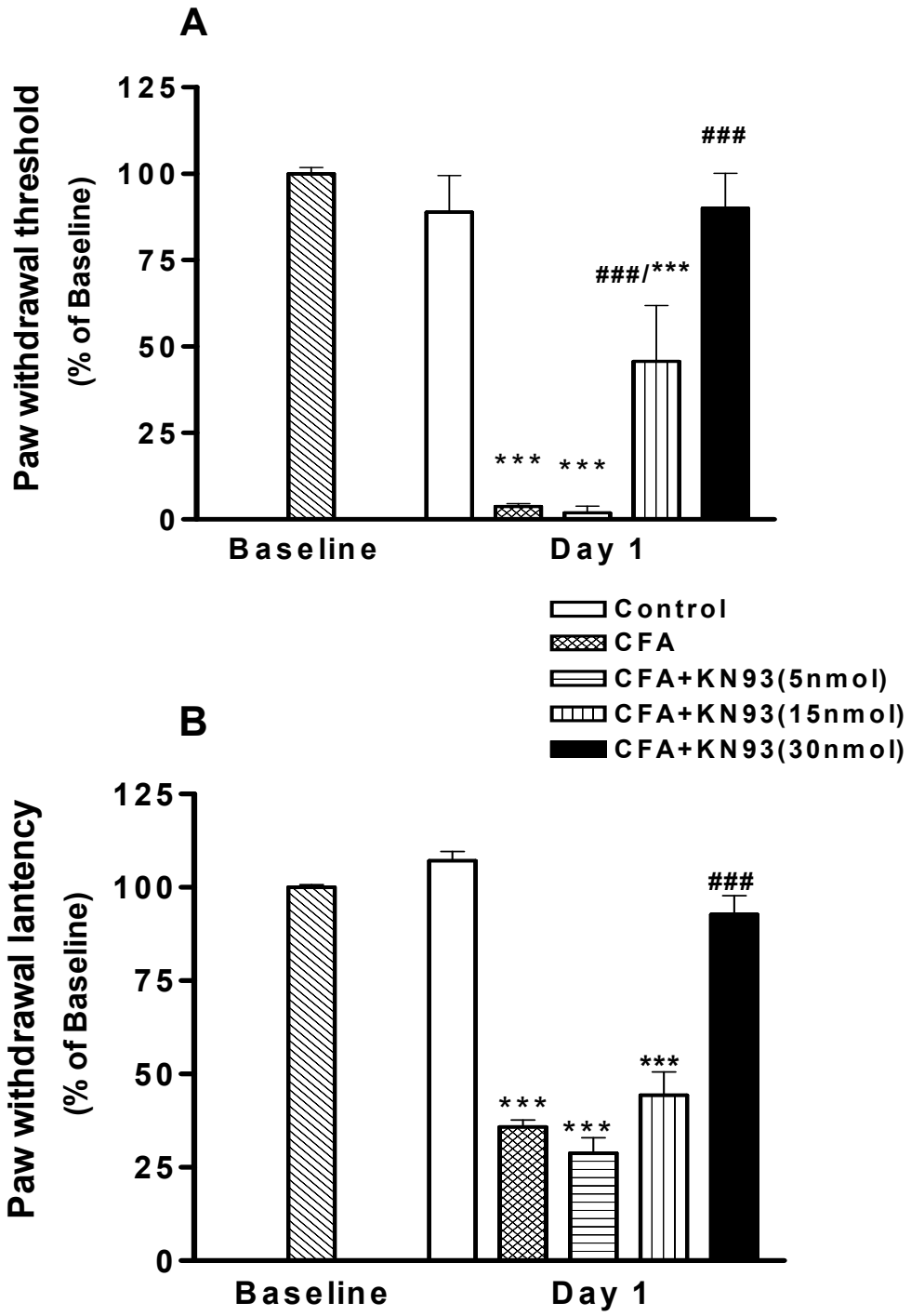


Figure 1. Prevention of CFA-induced mechanical allodynia (A) and thermal hyperalgesia (B) by KN93, a CaMKII inhibitor. Intraplantar injection of CFA (20 µl) induced mechanical allodynia and thermal hyperalgesia in mice. To prevent the development of CFA-induced allodynia and hyperalgesia, groups of eight mice were pretreated with KN93 (5, 15, and 30 nmol i.t.) 30 min before the injection of CFA, and pain testing was conducted 24 h later. Data are expressed as mean ± S.E.M. ***, $p < 0.001$ compared with the control group; ###, $p < 0.001$ compared with the CFA group, $n = 8$ for each group. ED50 values for KN93 were estimated to be 15.9 (blocking allodynia) and 19.4 nmol (blocking hyperalgesia)

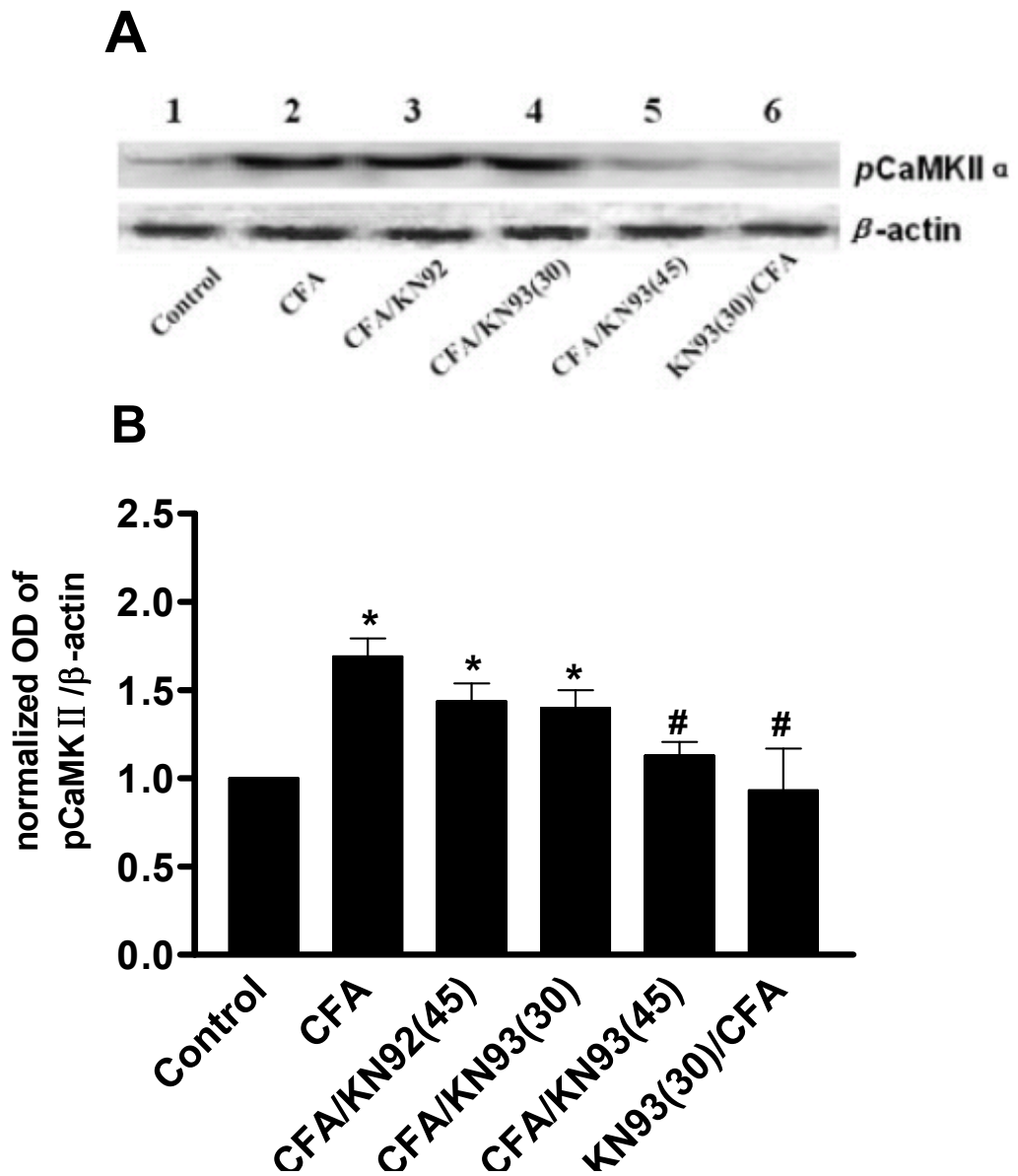


Figure 2. Suppression of CFA-induced spinal CaMKII activity by KN93, but not KN92. (A), intraplantar injection of CFA (20 μl) significantly increased CaMKII activity (pCaMKII) in the lumbar spinal cord compared with the saline-injected group (Control). This CFA-induced spinal CaMKII activity was reduced by an acute treatment (i.t.) with KN93 at a dose of 45 nmol [lane 5, CFA/KN93(45)], but not 30 nmol [lane 4, CFA/KN93(30)] or with KN92 at 45 nmol (lane 3, CFA/KN92). On the other hand, pretreatment with KN93 (30 nmol i.t.) was able to reduce CFA-induced CaMKII activity [lane 6, KN93(30)/CFA]. (B), histograph, expressed as mean ± S.E.M., was constructed from the representative figure shown and three other experiments. *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the CFA group, $n = 8$ for each group. Samples were taken immediately after the conclusion of behavioral studies (-2 h postdrug injections) on day 1 post-CFA.

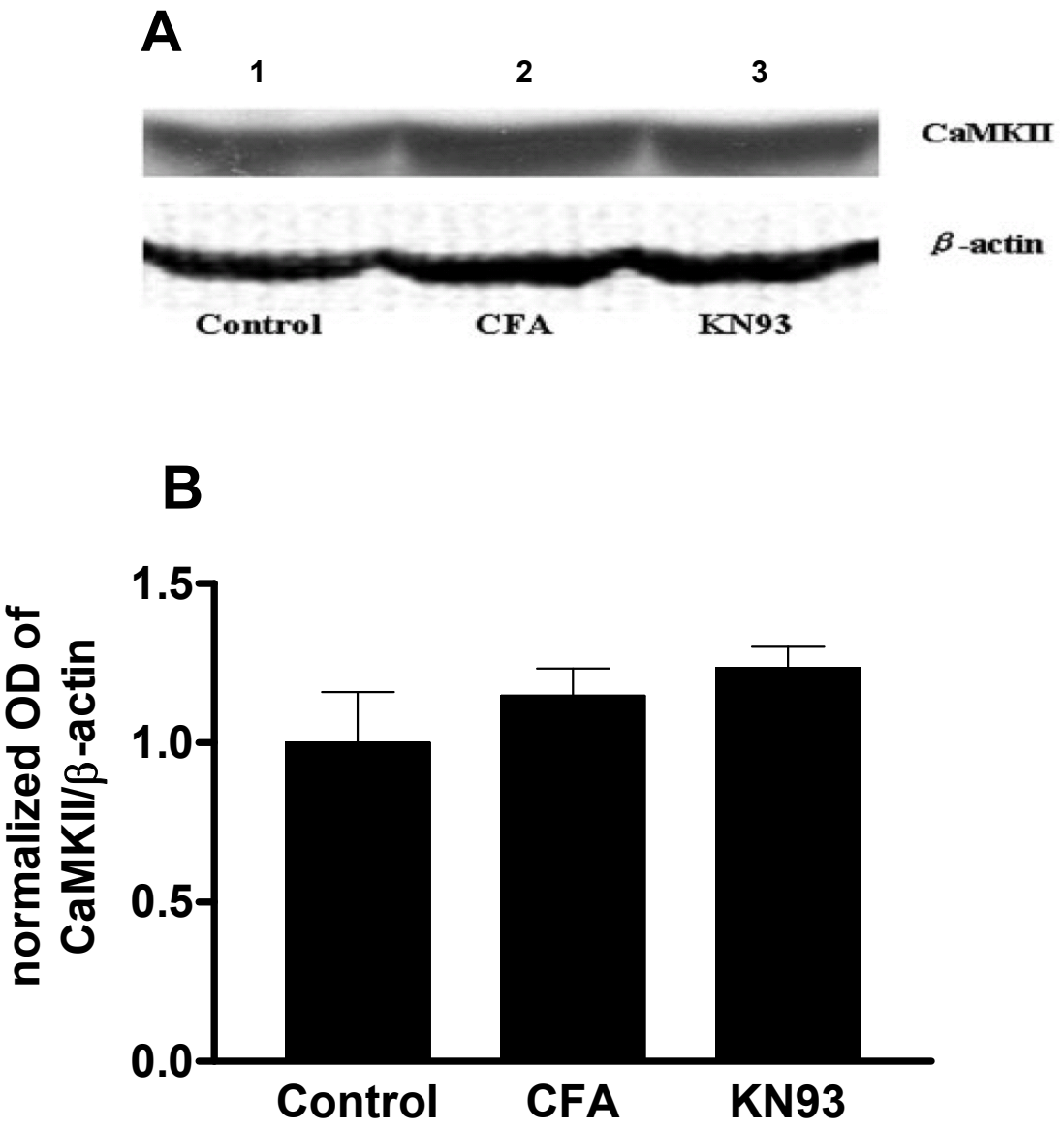


Figure 3. Spinal expression of CaMKII. Spinal CaMKII expression was determined in mice that received saline, CFA, or CFA + KN93 (30 nmol). Samples were taken 24 h post-CFA and CFA + KN93 treatments. Histograph, expressed as mean ± S.E.M., was constructed from the representative figure shown and three other experiments. No statistical difference was found.

3.2 Effect of Acute CaMKII Inhibition on CFA-Induced Inflammatory Pain

We next tested whether acute CaMKII inhibition could reverse established CFA-induced pain behavior. KN93 (45 nmol i.t.) reversed CFA-induced mechanical allodynia (Fig. 4A) and thermal hyperalgesia (Fig. 4B) on day 1 post-CFA injection ($p < 0.001$ compared with the CFA group, $n = 8$). KN93 at a lower dose (30 nmol) was partially effective ($p < 0.01$ compared either with the control or the CFA group, $n = 8$) in reversing CFA-induced thermal hyperalgesia but not mechanical allodynia. KN93 at an even lower dose (15 nmol) did not affect CFA-induced allodynia or hyperalgesia (Fig. 4). CFA-increased CaMKII activity was significantly attenuated by KN93 only at the higher dose (45nmol) (Fig. 2, lane 5, $p < 0.05$ compared with the CFA group, $n = 4$) but not at the lower dose (30 nmol) (Fig. 2, lane 4, $p > 0.05$). Therefore, KN93 dose-dependently reversed mechanical allodynia and thermal hyperalgesia, consistent with the inhibitor's action on CaMKII activity. ED_{50} values were estimated to be 30.2 (antiallodynia) and 29.5 nmol (antihyperalgesia). On day 3 post-CFA, allodynia and hyperalgesia had returned in the mice that were treated CFA + KN93 (data not plotted). In these mice, another dose of KN93 (45 nmol) on day 3 was able to again reverse the allodynia and hyperalgesia (Fig. 4). Treatment with KN92 (45 nmol, i.t.) did not affect CFA-induced CaMKII activation (Fig. 2, lane 3, $p > 0.05$ compared with the CFA group, $n = 4$), allodynia, or hyperalgesia (Fig. 4, $p > 0.05$ compared with the CFA group, $n = 8$). Neither KN93 nor KN92 (45 nmol i.t.) caused changes in gross behavior or nociception baseline in naive mice (Fig. 5).

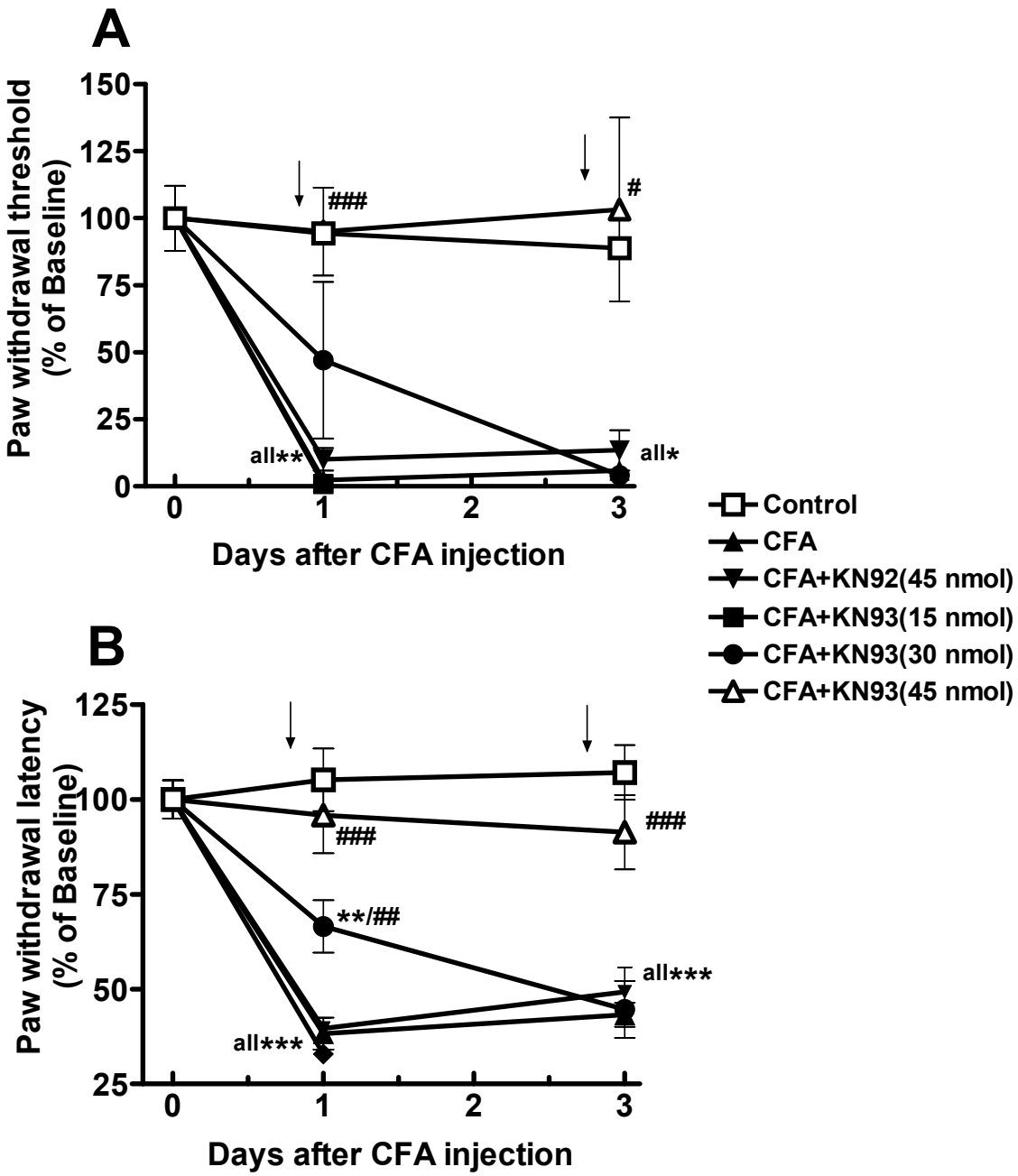


Figure 4. Reversal of CFA-induced mechanical allodynia (A) and thermal hyperalgesia (B) by acute inhibition of CaMKII. KN93, but not KN92 (45 nmol), administered i.t. was able to reverse the established CFA-induced mechanical allodynia and thermal hyperalgesia in a dose-dependent manner on day 1. On day 3, mechanical allodynia and thermal hyperalgesia had returned in the CFA + KN93 groups; however, another treatment with KN93 (45 nmol) was again able to acutely reverse the mechanical allodynia and thermal hyperalgesia. Data are expressed as mean ± S.E.M. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with the control group; #, $p < 0.05$, ##, $p < 0.01$, ###, $p < 0.001$ compared with the CFA group, $n = 8$ for each group. Arrows indicate the time when KN93, KN92, or normal saline was injected. ED50 values for KN93 were estimated to be 30.2 (antiallodynia) and 29.5 nmol (antihyperalgesia).

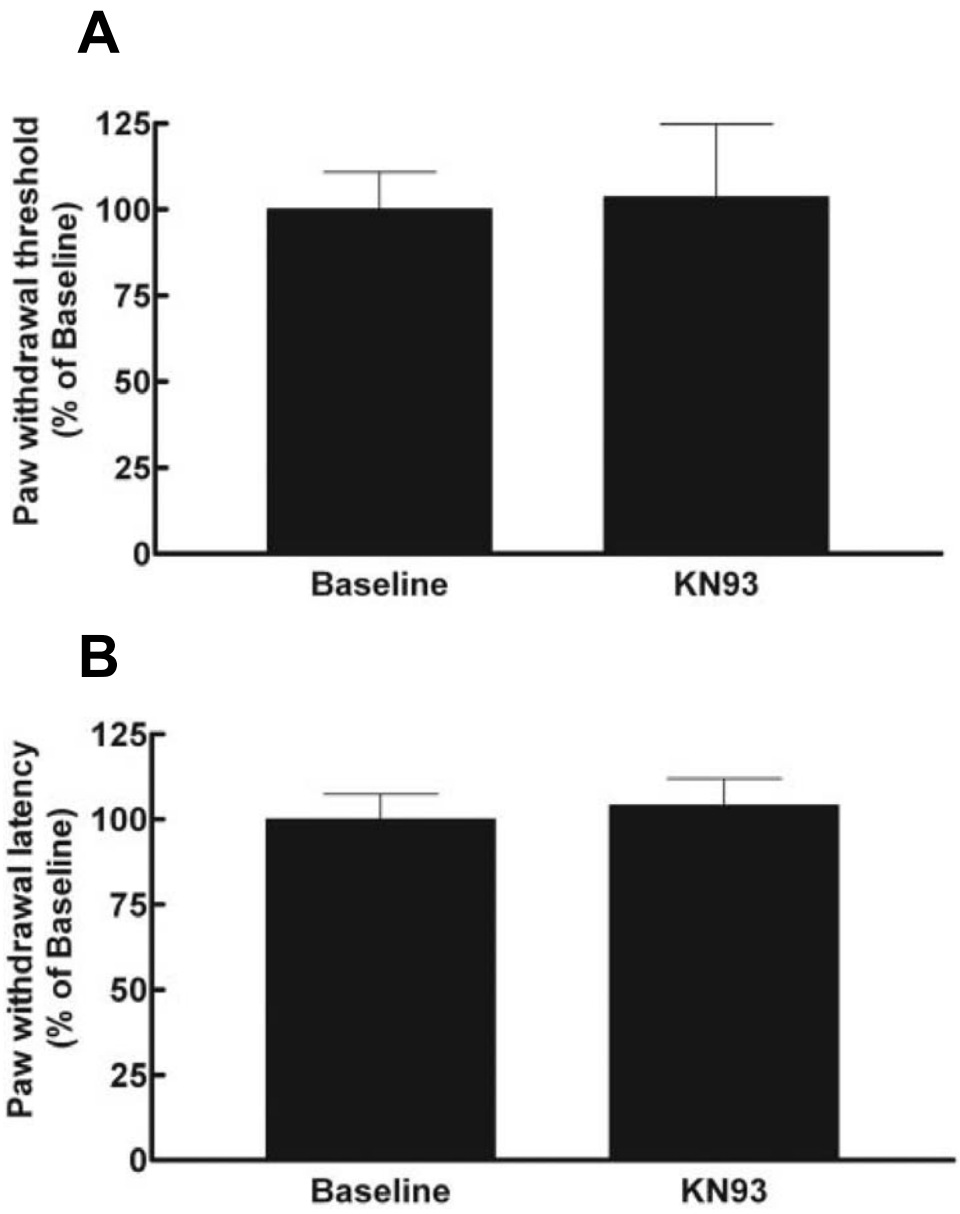


Figure 5. Effect of KN93 on thermal and mechanical nociception baseline in normal mice. KN93 (45 nmol i.t., 2 h postinjection) did not alter the paw-withdrawal threshold to mechanical stimulus (A) and the paw-withdrawal latency to radiant heat (B). Data are expressed as mean ± S.E.M., n = 8.

To determine a more precise time course of the action of KN93 (i.t.) in reversing CFA-induced allodynia and hyperalgesia, another series of experiments was performed in which the thermal and mechanical sensitivities were monitored for up to 24 h post-KN93 in CFA-treated mice (Fig. 6). The effect of KN93 (30 and 45 nmol) started at 30 min, the first testing time point, and peaked at 2 h in both allodynia and hyperalgesia studies. Antihyperalgesic effect of KN93 (30 and 45 nmol) lasted for at least 4 h, whereas the antiallodynic action was marginally significant at 4 h only for the highest dose ($p < 0.05$ compared with the CFA group, $n = 8$). KN93 at 15 nmol did not affect either thermal or mechanical sensitivity. At the time of the peak effect (2 h), ED_{50} values were estimated to be 30.3 (antiallodynia) and 28.7 nmol (antihyperalgesia). By the next day (24 h), all KN93 and CFA-treated mice achieved essentially the same thermal and mechanical sensitivities compared with the mice treated with CFA alone ($p < 0.001$ compared with the control, $n = 8$).

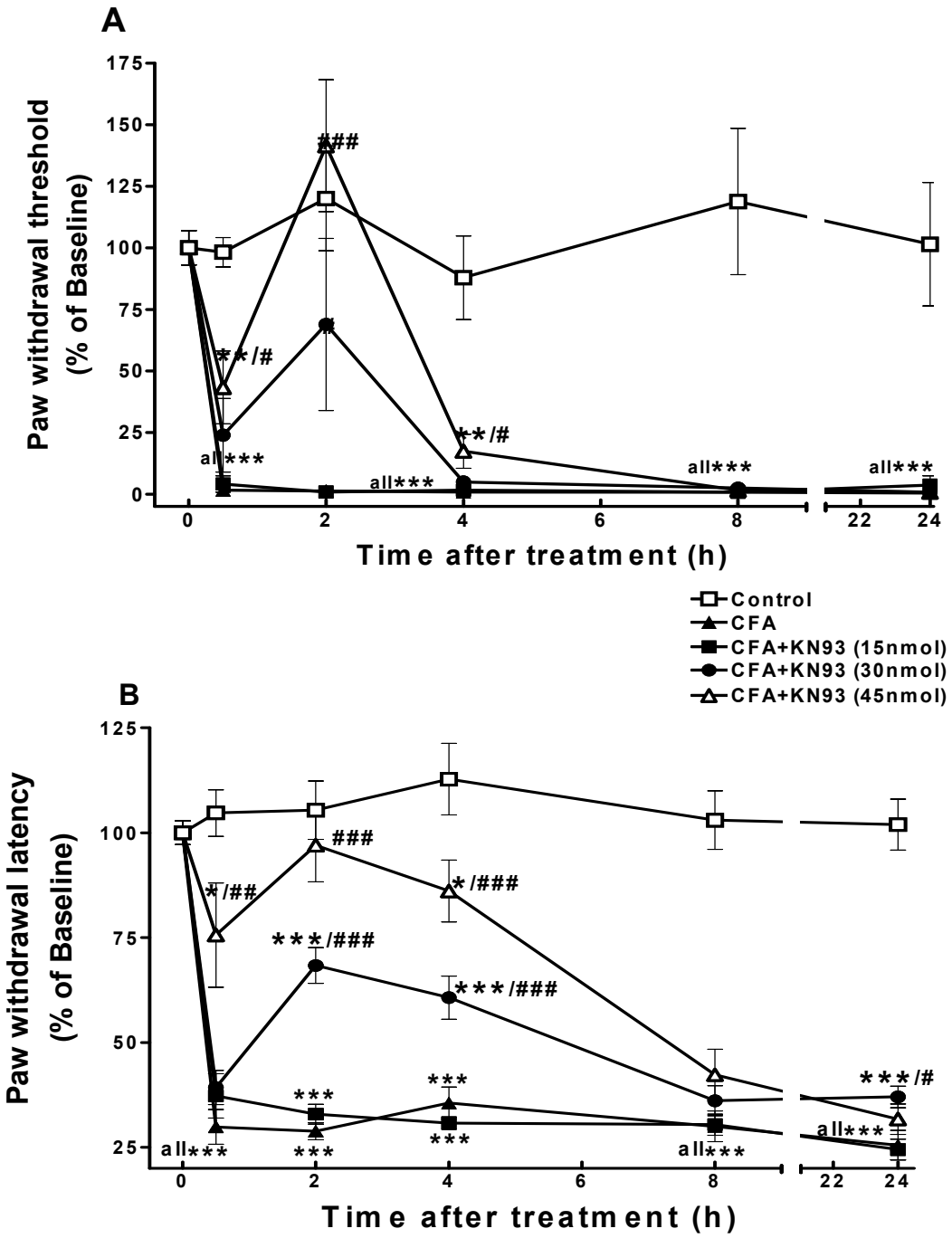


Figure 6. Time course of the reversal of CFA-induced mechanical allodynia (A) and thermal hyperalgesia (B) by the acute inhibition of CaMKII. KN93 dose-dependently reversed the established CFA-induced mechanical allodynia and thermal hyperalgesia 24 h post-CFA. The action of KN93 started within 30 min, peaked at 2 h, and lasted for at least 2 to 4 h. Data are expressed as mean ± S.E.M. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with the control group; #, $p < 0.05$, ##, $p < 0.01$, ###, $p < 0.001$ compared with the CFA group, n = 8 for each group. At a 2-h time point, ED50 values for KN93 were estimated to be 30.3 (antiallodynia) and 28.7 nmol (antihyperalgesia).

3.3 Trifluoperazine Reversed CFA-Induced Hyperalgesia and Allodynia

Trifluoperazine is a clinically used, orally available antipsychotic drug. Previously, we found that trifluoperazine also inhibited spinal and superspinal pCaMKII (Tang et al., 2006c). In the current study, we further verified that trifluoperazine inhibited CaMKII activity, with an EC_{50} = 14.4±1.1μM, in an in vitro CaMKII enzymatic assay (Fig. 7A). This drug, therefore, not only provides another test of our hypothesis but more importantly may ultimately provide a drug therapy targeting the CaMKII signaling pathway. Trifluoperazine (0.5 mg/kg i.p.) completely reversed mechanical allodynia (Fig. 8A) and thermal hyperalgesia (Fig. 8B) induced by CFA ($p < 0.001$ compared with the CFA group, n = 8). Correlating with its behavioral effect, trifluoperazine was found to suppress CFA-induced increase of spinal CaMKII activation (pCaMKII) in mice (Fig. 7B). The drug exhibited a partial effect in alleviating mechanical allodynia at a lower dose (0.25 mg/kg i.p.) ($p < 0.05$ compared with the CFA group, n = 8). At an even lower dose (0.1 mg/kg i.p.) trifluoperazine had no effect on CFA-induced hyperalgesia or allodynia (Fig. 8). Therefore, trifluoperazine was able to reverse CFA-induced inflammatory pain in a dose-dependent manner. ED_{50} values were estimated to be 0.3 (antiallodynia) and 0.3 mg/kg (antihyperalgesia).

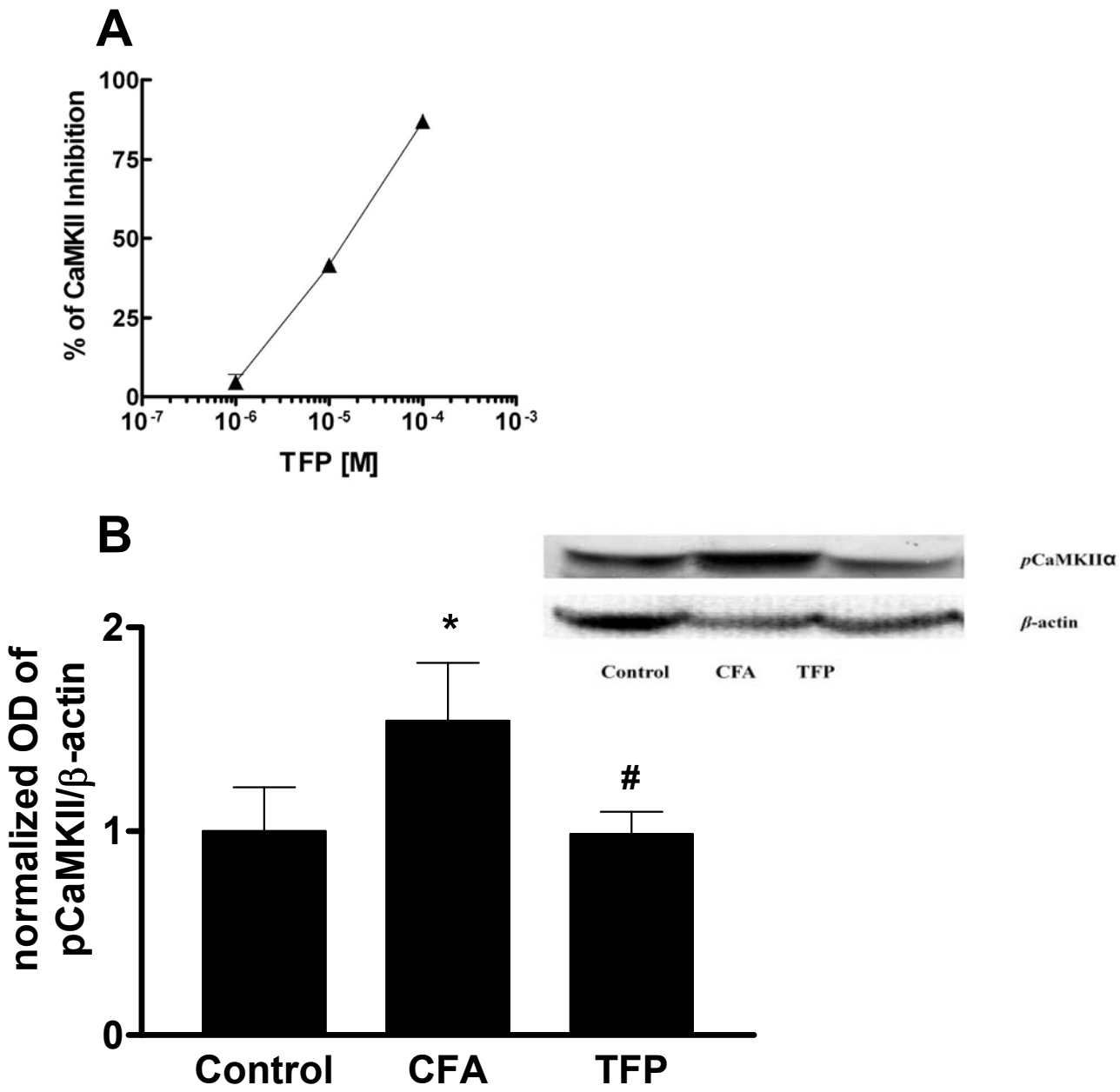


Figure 7. Inhibition of CaMKII by trifluoperazine. A, trifluoperazine (TFP) inhibited CaMKII activity in an enzymatic reaction using autocamtide-2, a selective CaMKII substrate. The amount [γ-^{32}P] incorporation was used to determine the phosphorylation of autocamtide-2 by CaMKII. Trifluoperazine dose-dependently inhibited CaMKII activity. EC_{50} = 14.4 ± 1.1 μM. B, TFP suppressed CFA-induced spinal CaMKII activity. Intraplantar injection of CFA (20 μl) significantly increased CaMKII activity (pCaMKII) in the lumbar spinal cord 24 h postinjection. This CFA-induced spinal CaMKII activity was reduced by an acute treatment with TFP (0.5 mg/kg i.p.). Histograph, expressed as mean ± S.E.M., was constructed from the representative figure shown and three other experiments. *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the CFA group, n = 8 for each group. Samples were taken 1 h after the injection of TFP on day 1 post-CFA.

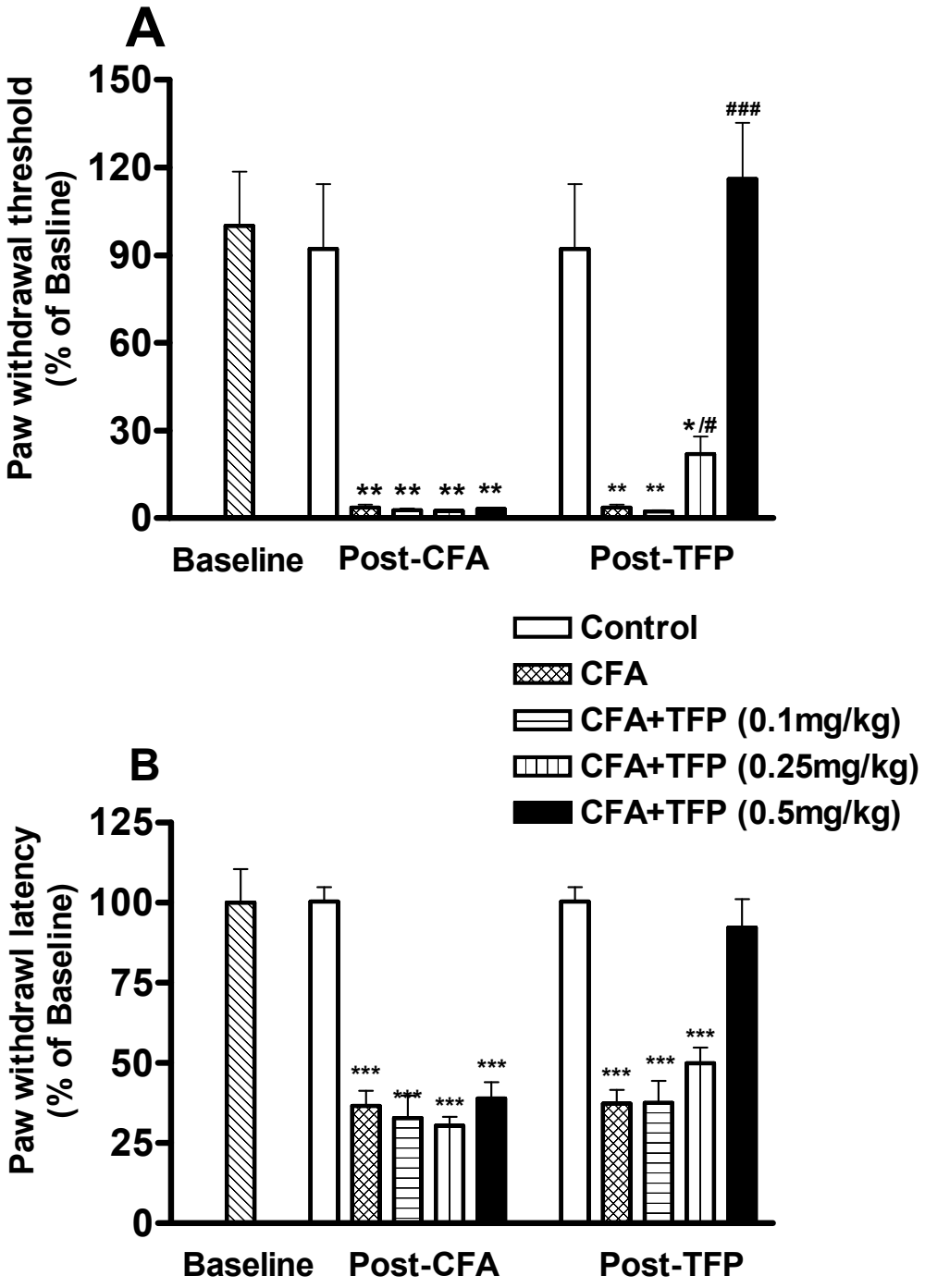


Figure 8. Reversal of CFA-induced mechanical allodynia (A) and thermal hyperalgesia (B) by trifluoperazine (TFP). Trifluoperazine (0.1– 0.5 mg/kg i.p.) was administered 1 day post-CFA injection (20 µl i.pl.). Nociception testing was performed before (Post-CFA) or 2 h after the administration of trifluoperazine (Post-TFP). Data are expressed as mean ± S.E.M. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with the control group; #, $p < 0.05$, ###, $p < 0.001$ compared with the CFA group, $n = 8$ for each group. ED50 values for trifluoperazine were estimated to be 0.3 (antiallodynia) and 0.3 mg/kg (antihyperalgesia).

To examine the time course of the action of trifluoperazine in reversing CFA-induced allodynia and hyperalgesia, thermal and mechanical sensitivities were monitored for up to 48 h in CFA-pretreated mice that received a single dose of trifluoperazine (0.5 mg/kg i.p.) (Fig. 9). Trifluoperazine showed a rapid onset of reversing the established CFA-induced thermal hyperalgesia, even at the first time point (30 min). The antihyperalgesic effect peaked at 2 to 4 h and lasted for at least 8 h. In the mechanical sensitivity experiments, the drug not only exhibited potent antiallodynic action, it further produced analgesia ($p < 0.05$ compared with the control at the time of the peak effect = 4 h, $n = 8$). The potential analgesic action was followed up in experiments in which naive mice were given a single intraperitoneal injection of trifluoperazine (0.5 mg/kg). During the first 30 to 60 min, trifluoperazine did not alter baseline mechanical or thermal nociception (Fig. 10, $p > 0.05$ compared with the predrug baseline, $n = 8$). Given that antiallodynic and antihyperalgesic actions of trifluoperazine had a rapid onset (30 min), these data suggest that its antiallodynic and antihyperalgesic actions do not entirely depend on its analgesia. At 2 to 4 h postinjection, trifluoperazine produced significant analgesia by itself, although the magnitude was smaller than that of antiallodynic and antihyperalgesic actions. In addition, the duration of action was shorter than that of antiallodynic and antihyperalgesic actions. At 8 h postinjection when antiallodynic and antihyperalgesic actions were still observed, trifluoperazine produced a weak but significant thermal hyperalgesic effect.

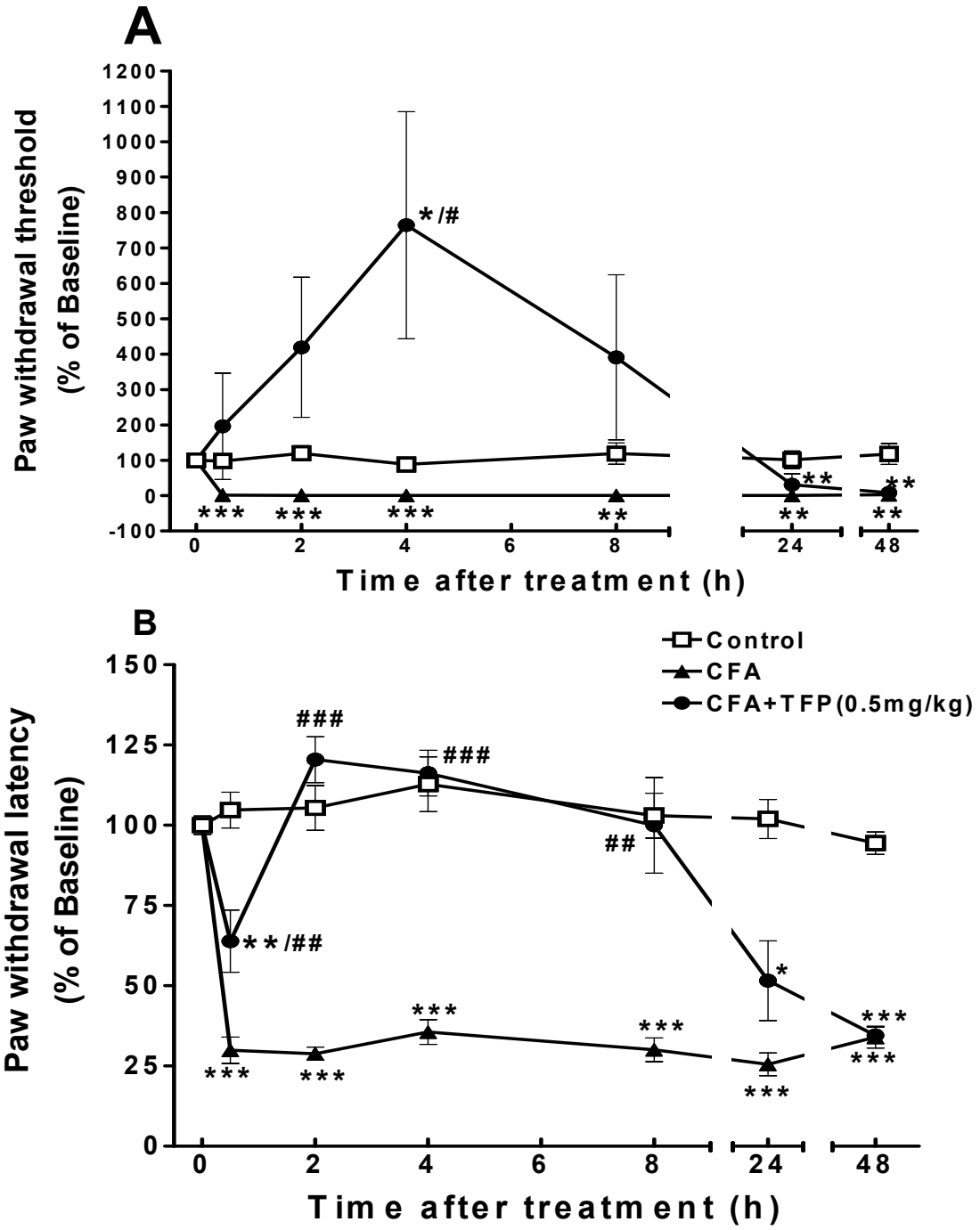


Figure 9. Time course of the reversal of CFA-induced mechanical allodynia (A) and thermal hyperalgesia (B) by trifluoperazine (TFP). Thermal and mechanical sensitivities were monitored for 48 h in the CFA-pretreated mice that received a single dose of trifluoperazine (0.5 mg/kg i.p.). Trifluoperazine showed a rapid onset of antihyperalgesic effect, even at the first time point (30 min). The antihyperalgesic effect peaked at 2 to 4 h and lasted for at least 8 h. In the mechanical sensitivity experiments, the drug not only exhibited potent antiallodynic action, it also exhibited analgesia that last for at least 8 h. Data are expressed as mean ± S.E.M. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with the control group; #, $p < 0.05$, ##, $p < 0.01$, ###, $p < 0.001$ compared with the CFA group, n = 8 for each group.

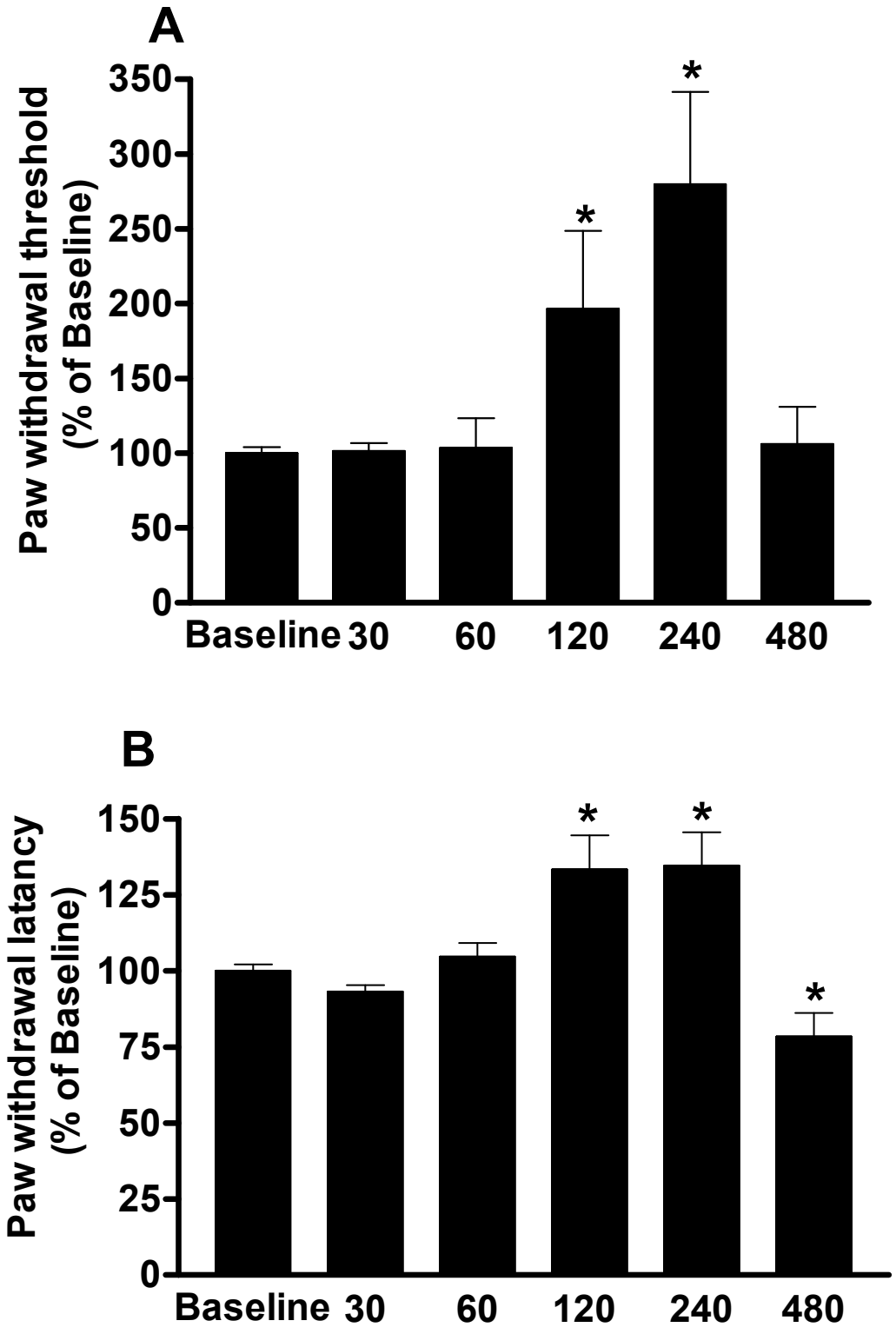


Figure 10. Effect of trifluoperazine on nociceptive baseline in naive mice. During the first 30 to 60 min, trifluoperazine (0.5 mg/kg i.p.) did not alter baseline mechanical (A) or thermal (B) nociception. At 2 to 4 h post trifluoperazine, the drug produced significant analgesia. A weak but significant thermal hyperalgesic effect was observed at 8 h. Data are expressed as mean ± S.E.M. *, $p < 0.05$, **, $p < 0.01$, compared with the baseline, $n = 8$.

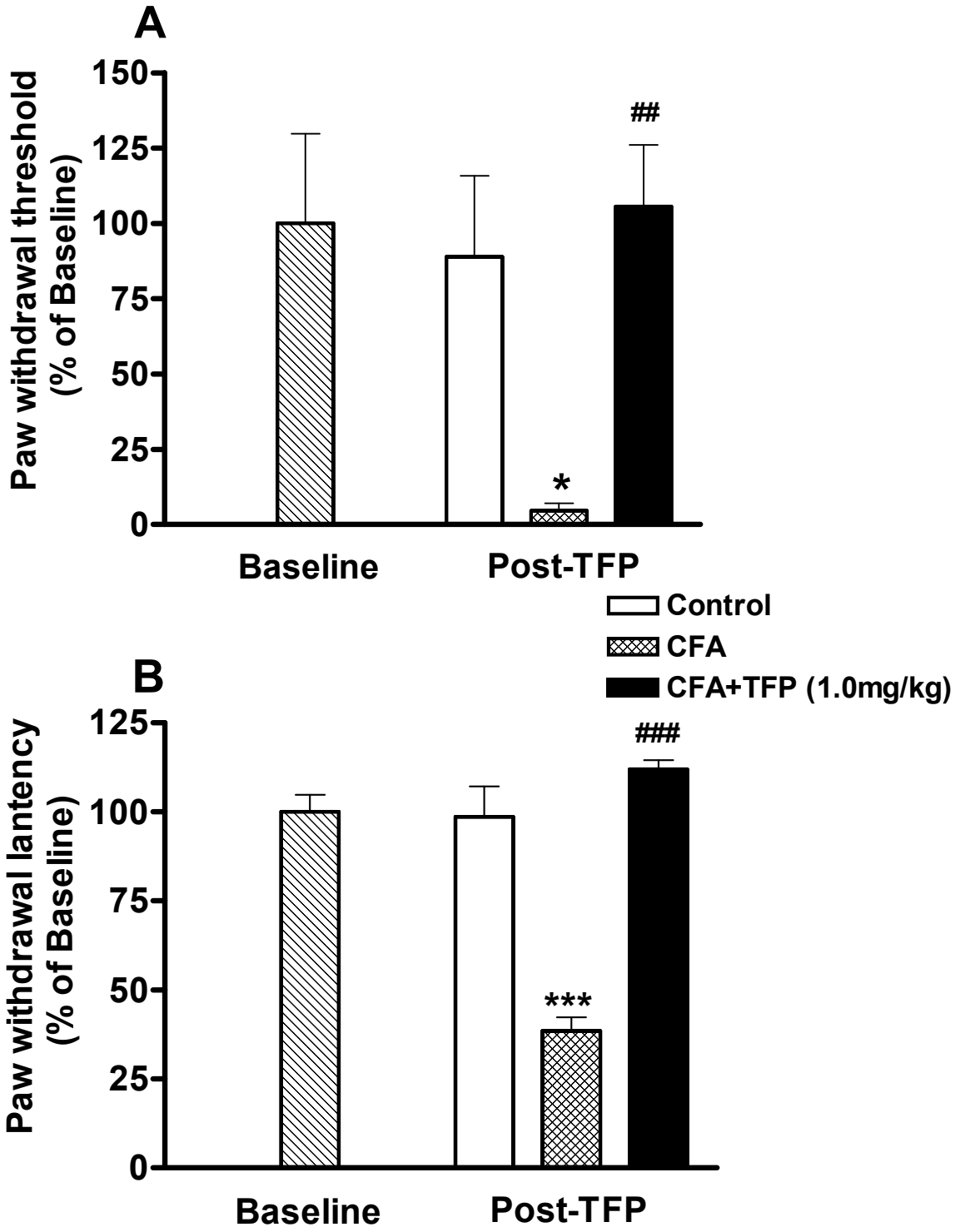


Figure 11. Reversal of CFA-induced mechanical allodynia (A) and thermal hyperalgesia (B) by oral trifluoperazine. Trifluoperazine (1 mg/kg, gastric gavage) was administered 1 day post-CFA injection (20 µl i.pl.). Nociception testing was performed 2 h after the administration of trifluoperazine (Post-TFP). Trifluoperazine significantly reversed CFA-induced mechanical allodynia and thermal hyperalgesia. Data are expressed as mean ± S.E.M. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with the control group; ##, $p < 0.01$, ###, $p < 0.001$ compared with the CFA group, n = 6 for each group.

Because trifluoperazine can be given orally, we further tested whether oral trifluoperazine (1 mg/kg, gastric gavage) was effective. Indeed, the drug significantly reversed CFA-induced mechanical allodynia and thermal hyperalgesia (Fig. 11).

4. Discussion

In this study, the role of CaMKII in an experimental model of inflammatory pain was investigated. In the first series of experiments, the role of CaMKII in the generation of inflammatory pain induced by intraplantar CFA was analyzed. Treatment with the CaMKII inhibitor KN93 (30 or 15 nmol i.t.) shortly before the administration of CFA fully (30 nmol) or partially (15 nmol) prevented the development of hyperalgesia and allodynia when tested 24 h post-CFA. These data are in agreement with previous reports that KN93 (i.t. infusion) could prevent CCI- and inferior alveolar nerve transection-induced pain (Dai et al., 2005; Ogawa et al., 2005).

The main hypothesis that CaMKII is required for the maintenance of inflammatory pain was further tested. Such studies are critical for designing new pain therapies based on the CaMKII signaling pathways, because most patients present with long-lasting abnormal pain after, not before, nerve or tissue injuries. A previous study found KN93 (given i.t. 7-day post injury, 0.25 μg/μl/h via an Alzet pump for 7 days) to be ineffective in reversing already established neuropathic pain after CCI (Dai et al., 2005). In contrast, it was reported that KN93 (120 pmol i.t.) and myristoylautocamtide 2-related inhibitory peptide (1 nmol i.t.) effectively reversed CCI-induced neuropathic pain in mice, although the exact experimental details were

not provided (Garry et al., 2003). Our data demonstrated that KN93 was effective in reversing established CFA-induced inflammatory pain. The discrepancy could have been caused by different experimental models of pain or animals used in these studies. An alternative explanation may be related to the degree of CaMKII inhibition. Compared with the dose for preventing CFA-induced inflammatory pain, a higher dose of KN93 was necessary to reverse CFA-induced hyperalgesia and allodynia. When we attempted to reverse CFA-induced hyperalgesia and allodynia with KN93 at lower doses (15–30 nmol), the drug was partially effective or not effective. As the dose was increased to 45 nmol, CFA-induced inflammatory pain was acutely reversed. The degree of CaMKII inhibition in relation to the CaMKII activity in the pain state was further supported by biochemical data indicating that CaMKII activity was upregulated considerably in the pain state (Fig. 2, lane 2 compared with lane 1). Furthermore, KN93 at the higher dose (45 nmol; Fig. 2, lane 5), but not at the lower dose (30 nmol; Fig. 2, lane 4), was effective in reducing the up-regulated CaMKII activity. As an agent to prevent CFA-induced pain, KN93 at 30 nmol was sufficient to prevent the up-regulation of CaMKII activity (Fig. 2, lane 6 compared with lane 2). The increase in pCaMKII was largely caused by the increased activity, as CaMKII expression was not significantly increased by the pretreatment with CFA or CFA + KN93. Therefore, different doses of CaMKII inhibitors were required to produce sufficient CaMKII inhibition depending on the activity of CaMKII that was heightened in the chronic pain states. A similar observation has been made previously in the studies of opioid tolerance in which higher doses of CaMKII inhibitors were required to achieve

sufficient CaMKII inhibition when the degree of opioid tolerance was increased (Tang et al., 2006a). Data from our and two other laboratories (Garry et al., 2003; Dai et al., 2005; Ogawa et al., 2005) have so far suggested an important role of CaMKII in chronic pain, which was not supported by a study employing CaMKIIα (T286A) mutant mice (Zeitz et al., 2004). These mice express a mutant form of CaMKII that cannot be auto-phosphorylated and activated. Whereas the second phase of formalin-induced paw-licking behavior was significantly reduced in CaMKIIα(T286A) mutant mice, wild-type and mutant mice showed similar CFA- or formalin-induced thermal and mechanical pain thresholds (Zeitz et al., 2004). The exact reason for the discrepancy is not known; however, two different approaches are used in these studies to chemically inhibit or genetically eliminate the normal function of CaMKII. Interpretation of experiments applying chemical inhibitors can be compromised by the potential lack of selectivity of the inhibitors used. On the other hand, mouse genetic mutation studies can suffer from problems association with gene manipulation, such as unmatched genetic background, compensatory changes, or other nonspecific genomic effects.

The acute action of CaMKII inhibitors in reversing established pain was further supported by the experiments employing the antipsychotic drug trifluoperazine, which is a calmodulin inhibitor and suppresses CaMKII activity (Tang et al., 2006c). Therefore, trifluoperazine not only presents a unique opportunity to test our hypothesis but more importantly may provide a drug candidate for alleviating chronic pain in clinical settings. Systemic trifluoperazine (i.p.) dose-dependently reversed mechanical allodynia and thermal hyperalgesia in CFA-treated mice. The drug was also effective

given orally. These data suggested that the action of CaMKII inhibitors was not limited to spinal intervention. Trifluoperazine (i.t.) has been previously reported to produce either analgesia (at low doses) or hyperalgesia (at a high dose) in a formalin-induced inflammatory pain model (Golbidi et al., 2002). The doses we used would have been considered as “low doses”, although the exact conversion was not possible due to different routes of administration. Our data suggested that the analgesia/hyperalgesia balance might also depend on the duration of treatment. However, the antiallodynic and antihyperalgesic actions of trifluoperazine in the current study could not be fully attributed to its analgesic action, because the former had an earlier onset, larger magnitude of effect, and longer duration of action. In fact, at 8 h postinjection when analgesia had disappeared and trifluoperazine produced a weak thermal hyperalgesic effect in naive mice, antiallodynic and antihyperalgesic actions persisted in CFA-treated mice.

How persistent CaMKII activation is achieved in pain states is puzzling, because one would probably expect desensitization of a kinase after prolonged activation. One plausible mechanism may be through the interaction of CaMKII with NMDA receptors. It has been demonstrated that CaMKII can phosphorylate NMDA receptors and enhance receptor function (McGlade-McCulloh et al., 1993; Lau and Huganir, 1995). Phosphorylation of the NMDA receptor is a key means to regulate the function of this ligand-gated ion channel, which is most permeable to Ca^{2+}. Phosphorylation of NMDA receptors by CaMKII has been shown to enhance the NMDA receptor function, leading to the influx of Ca^{2+} through the channels (Kitamura

et al., 1993). Therefore, activation of CaMKII as a result of inflammation and nerve injury can potentially increase the activity of the NMDA receptors leading to Ca^{2+} influx. Increased cytosolic Ca^{2+} ions bind and change the conformation of CaM, which in turn leads to the activation of more CaMKII (Strack et al., 1997). Therefore, a feed-forward loop may exist between CaMKII and the NMDA receptors in the chronic pain state.

Indeed, inflammatory injury has been shown to increase levels of glutamate in the spinal dorsal horn (Sluka and Westlund, 1992), and this was blocked by NMDA receptor antagonists (Sluka and Westlund, 1993). It has also been demonstrated that NMDA receptor antagonists administered before inflammatory or peripheral nerve injuries suppress or delay the onset of hyperalgesia and attenuate fully developed hyperalgesia when administered after the injury (Zhang et al., 1998; Dai et al., 2005). Overexpression of the NR2B subunit of the NMDA receptor causes increased inflammatory mechanical allodynia, whereas knockdown of spinal NMDA receptors with antisense oligonucleotides prevents the expression of chemically induced hyperalgesia (Garry et al., 2000; Wei et al., 2001). Therefore, CaMKII may work in concert with the NMDA receptors in the development and maintenance of the neural events leading to hyperalgesia.

In conclusion, our findings suggest the critical involvement of CaMKII in the process of maintaining and/or inducing persistent inflammatory pain. Blocking the CaMKII signaling pathway may provide a useful therapeutic target for the treatment of chronic pain. Trifluoperazine at relatively (to its antipsychotic effect) low doses was found to be highly efficacious in reversing the CFA-induced inflammatory pain.

Although the drug has multiple pharmacologic effects and is not expected to be selective at CaMKII, it is approved by the United States Food and Drug Administration as an orally available antipsychotic drug that has been used in humans for many years. Therefore, we propose that trifluoperazine should be tested in clinical settings for the treatment of chronic pain.

III. ACUTE INHIBITION OF Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II REVERSES EXPERIMENTAL NEUROPATHIC PAIN

1. Introduction

Peripheral nerve injury often leads to pathologic persistent pain that can be presented with spontaneous pain, hyperalgesia, and allodynia (Dubner and Ruda, 1992; McMahon et al., 1993; Willis, 2001). Central sensitization, as a result of neuronal plasticity is considered a pivotal mechanism leading to the development of hyperalgesia and allodynia (Woolf, 2007; Pezet et al., 2008). Although the specific pathways have yet to be elucidated, the N-methyl-d-aspartate (NMDA) receptors have been implicated consistently in the development of central sensitization (Woolf and Thompson, 1991; Wang et al., 2005). Not surprisingly, blockade of the NMDA receptors has been shown to prevent or disrupt pain behaviors in experimental models of neuropathic pain e.g. (Seltzer et al., 1991; Dickenson et al., 1997). Activation of the NMDA receptors leads to increased Ca^{2+} influx into cytosol. This increased Ca^{2+} influx initiates cascades of intracellular signaling events involving Ca^{2+} and various protein kinases (Womack et al., 1988). Numerous studies have suggested that the Ca^{2+}-mediated cell signaling pathways are crucial to the nociception e.g.,(Saegusa et al., 2001; Kim et al., 2003). In particular, Ca^{2+} activates protein kinase C, specific isoforms of which have been demonstrated to be important in studies employing various experimental neuropathic pain models (Malmberg et al., 1997; Hua et al., 1999; Dina et al., 2000).

Limited studies have been reported for another major intracellular protein kinase that is involved in Ca^{2+} signaling, namely, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Accounting for approximately 1 to 2% of total brain protein mass, CaMKII is ubiquitously distributed in the central nervous system CaMKII is a serine/threonine protein kinase and, like PKC, is activated by Ca^{2+} signaling (Lisman et al., 2002). A significant increase in intracellular Ca^{2+}, which is found in chronic pain states e.g., (Eisenach et al., 2005), first activates calmodulin (CaM) by binding to the Ca^{2+}-binding sites. This interaction leads to a change in the conformation of CaM. CaMKII is then activated by Ca^{2+}/CaM by switching to an active state on exposure to Ca^{2+}/CaM.

Although the pathway stated above has been well accepted, the actual role of CaMKII in neuropathic pain has yet to be established. The limited data in the literature are somewhat conflicting. One group found that, at very low doses, the CaMKII inhibitors, KN93 (120 pmol, i.t.) and myristoyl-autocamtide 2-related inhibitory peptide (1 nmol, i.t.), reversed chronic constriction injury (CCI)-induced neuropathic pain in mice (Garry et al., 2003). Another study reported a mixed effect that KN93 was capable of preventing, but not reversing, thermal hyperalgesia and mechanical allodynia after CCI (Dai et al., 2005). A third study found that nerve injury produced a similar enhancement of thermal and mechanical sensitivity in wild-type mice and CaMKIIα(T286A) mutant mice lacking the functional CaMKIIα (Zeitz et al., 2004). Our previous study, using a complete Freund's adjuvant (CFA)-induced persistent inflammation pain model, suggested that some of the conflicting results may be

reconciled by examining the degree of CaMKII activity in the pre- versus post-treatment states. Therefore, it is critical to ensure CaMKII inhibition as verified by biochemical methods in such studies involving a protein kinase inhibitor or activator (Luo et al., 2008). In this study, we tested the hypothesis that sufficient CaMKII inhibition is capable of acutely reversing the established spinal nerve ligation-induced pain behaviors. This was accomplished by examining the behavioral/biochemical correlation after treatments with CaMKII inhibitors. Investigating an acute action of CaMKII inhibition in chronic pain is critical not only for our understanding of the mechanisms but more importantly for designing useful drug therapies for neuropathic Pain.

2. Materials and Methods

2.1 Materials

KN92 [2-[N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine] and KN93 [2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)] amino-N- (4-chlorocinnamyl)-N-methylbenzylamine)] were purchased from Calbiochem (San Diego, CA). Trifluoperazine dihydrochloride and other chemicals were obtained from Sigma (St. Louis, MO).

2.2 Animals

Male ICR mice (25 ± 5 g, Harlan Laboratories, Indianapolis, IN) were provided food and water *ad libitum* with a 10:14h light/dark cycle. Mice were randomly divided

into experimental groups according to a computer generated randomization list. All animal experiments were carried out in accordance with the International Association for the Study of Pain (IASP) and the National Institute of Health Guidelines for the handling and use of laboratory animals after approval by the University of Illinois Institutional Animal Care and Use Committee.

2.3 Methods

2.3.1 Spinal nerve ligation

Spinal nerve ligation (SNL) operation was performed as published previously (Kim and Chung, 1992; Wang et al., 2001). Separate groups of eight mice had the left L5 and L6 spinal nerves tightly ligated distal to the dorsal root ganglion but before the fibers join to form the sciatic nerve; the sham operation consisted of the same surgery but without nerve ligations. Mice were tested for thermal hyperalgesia and tactile allodynia (see below) before and at different time points after the SNL operation.

2.3.2 Drug Administration

Intrathecal injection (i.t.) was performed as described previously (Hylden and Wilcox, 1980; Luo et al., 2008). A drug in a volume of 5 μl was given by percutaneous puncture through an intervertebral space at the level of the fifth or sixth lumbar vertebra. A lateral tail-flick was used to verify the success of injection. For acute reversal experiments, mice were given KN93 (15 - 45 nmol, i.t.), KN92 (45 nmol, i.t.), or trifluoperazine (0.1 - 0.5 mg/kg, i.p. or 0.1 - 1.0 mg/kg via gastric gavage) before

pain testing on day 5 after nerve ligation. In these studies, control mice received an equal volume of saline.

2.3.3 Mechanical Allodynia

Mechanical sensitivity was determined using calibrated von Frey filaments (Stoelting, Wood Dale, IL) as previously described (Chaplan et al., 1994; Tang et al., 2007a; Luo et al., 2008). These tests were performed before surgery to obtain the baseline sensitivity and daily after SNL by the same researcher. On the day of drug intervention, response to mechanical sensitivity was determined 0, 0.5, 2, 4, 8 and 24 h after the administration of a drug or vehicle. The up-down paradigm was used to determine 50% probability of paw withdrawal threshold (Dixon, 1980; Chaplan et al., 1994; Luo et al., 2008)

2.3.4 Thermal Hyperalgesia

The paw withdrawal latencies to radiant heat were tested the use of a plantar tester (model 7372, UGO BASILE, VA, Italy) as described previously (Hargreaves et al., 1988; Tang et al., 2007a; Luo et al., 2008). Mice were placed in a transparent cage on a glass floor. The radiant heat source was focused on the central portion of the plantar surface of the left hind paw and paw withdrawal latencies were recorded when the heat source was automatically turned off as a result of paw withdrawal. A cut-off time of 20 s was applied in order to prevent tissue damage.

2.3.5 Rotarod Test

To determine whether trifluoperazine at the doses used (administered intraperitoneally or by mouth) caused locomotor impairment in animals, the locomotor ability was tested with a rotarod treadmill (model series 8; IITC Life Science, Woodland Hills, CA). Mice were first trained to stay on the rotarod revolving at 16 rpm for two consecutive 120-s trials. On the next day, locomotor activity was tested 4 h (i.e., the peak antihyperalgesic/allodynic time) after the administration of trifluoperazine (0.5 mg/kg i.p. or 1.0 mg/kg p.o.) or saline (equal volume, intraperitoneally or by mouth). The duration when a mouse stays on the rotarod was recorded. A cutoff time (i.e., maximal test time) is 300 s.

2.3.6 Western Blotting Analysis

The lumbar sections of the spinal cord were quickly dissected from euthanized mice and separated into the ipsilateral and contralateral halves. Tissues were quickly frozen in acetone-dry ice solution and stored -80°C or immediately processed for Western blotting analysis as described previously (Tang et al., 2006a; Luo et al., 2008). In brief, tissues were homogenized in buffer A in the presence of phosphatase inhibitors (10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 μM okadaic acid) and protease inhibitors (0.05 mg/ml bestatin, 0.05 mg/ml leupeptin, 0.05 mg/ml pepstatin, and 0.1 mg/ml phenylmethylsulfonyl fluoride). The homogenates were incubated on a rotator for 1 h and centrifuged (45,000*g*) for 1 h at 4°C. Protein content in the supernatant was determined by a

modified Bradford method (Pierce Biotechnology, Rockford, IL). Samples were then separated by SDS-polyacrylamide gel electrophoresis and electrotransferred onto poly (vinylidene difluoride) membrane for Western blotting analyses. Antibodies including a rabbit anti-(T286/287)pCaMKII antibody (1:1000; Promega, Madison, WI), a rabbit anti-CaMKII antibody (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and a mouse anti-β-actin antibody (1:10,000; Sigma-Aldrich), and corresponding horseradish peroxidase conjugate secondary antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, UK) were used. The enhanced chemiluminescence signals (Pierce Biotechnology) were captured by a ChemiDoc imaging system and analyzed with use of the Quantity One program (Bio-Rad Laboratories, Hercules, CA). Ratios of the optical densities of pCaMKII or CaMKII to those of β-actin were calculated for each sample.

2.3.7 Statistical Analysis

A two-way repeated-measures analysis of variance was used to determine differences among groups. The Student-Newman-Keuls test was used as a post hoc test. Statistical significance was established at the 95% confidence limit.

3. Results

3.1 Effect of Acute CaMKII Inhibition on SNL-Induced Pain Behaviors

As expected, thermal hyperalgesia and mechanical allodynia developed

shortly after SNL. Within 48 h, withdrawal latencies to radiant heat and withdrawal thresholds to probing by von Frey filaments were significantly reduced in SNL-operated mice ($p < 0.001$ compared with the sham group, $n = 8$; Fig. 1). On day 5, when SNL-induced hyperalgesia and allodynia have fully developed, an acute administration of KN93 (30 and 45 nmol i.t.) 2 h before pain testing was able to reverse the established mechanical allodynia (Fig. 1A) and thermal hyperalgesia (Fig. 1B) ($p < 0.001$ compared with the SNL group or pretreatment baseline, $n = 8$). KN93 at the lowest dose used (15 nmol i.t.) did not show any effect on SNL-induced hyperalgesia or allodynia ($p > 0.05$ compared with the SNL group, $n = 8$). The same acute treatment with the kinase-inactive chemical analog KN92 (45 nmol i.t.) had no effect on SNL-induced hyperalgesia or allodynia (Fig. 1, $p > 0.05$ compared with the SNL group, $n = 8$).

To correlate the behavioral effects with the biochemical changes of CaMKII activity by KN93, not by KN92, the ipsilateral and contralateral lumbar sections of the spinal cord were dissected out on day 5 to determine the degree of CaMKII autophosphorylation (pCaMKII), which serves as a biomarker for CaMKII activity (Dai et al., 2005; Tang et al., 2006a; Luo et al., 2008). Compared with the sham operation, SNL significantly increased spinal pCaMKII on the ipsilateral side (Fig. 2A, $p < 0.001$, $n = 4$), but not on the contralateral side (Fig. 2C, $p > 0.05$, $n = 4$). The spinal expression of total CaMKII was not significantly altered (Fig. 2B), suggesting that the increased CaMKII activity was largely due to enhanced autophosphorylation (i.e., activation) of existing CaMKII. In SNL-operated mice, KN93 (30–45 nmol i.t.) significantly reversed

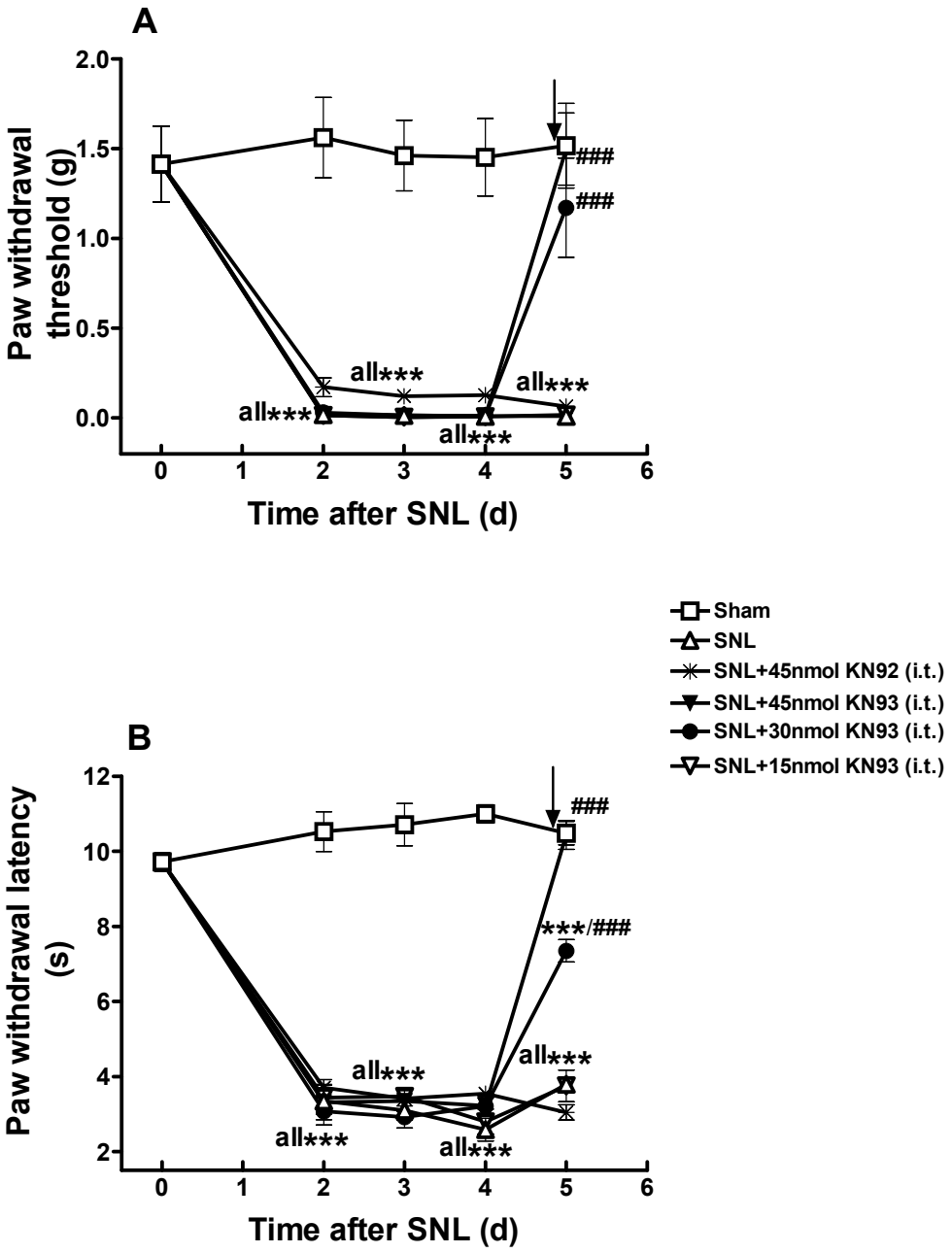
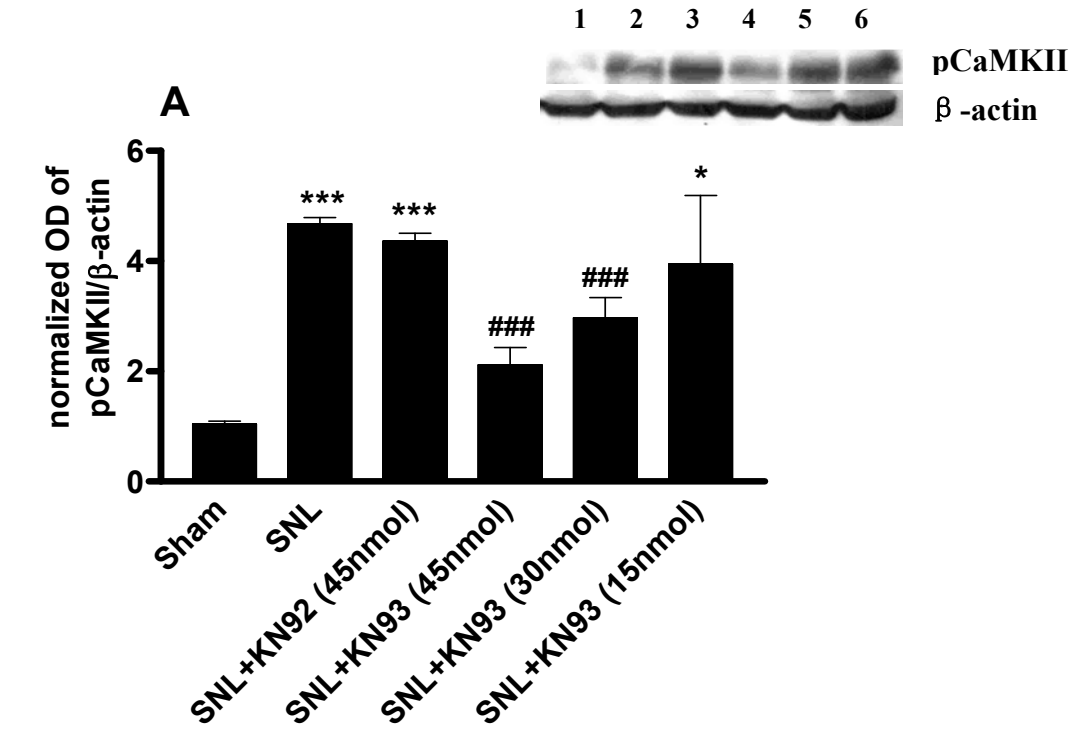


Figure 1. Reversal of spinal nerve ligation-induced mechanical allodynia (A) and thermal hyperalgesia (B) by KN93, a CaMKII inhibitor. The tight ligation of L5 and L6 spinal nerves (SNL) induced mechanical allodynia and thermal hyperalgesia in mice, which was absent in sham-operated mice. KN93, KN92 or saline were administered *(i.t.)* 2h before mechanical and thermal sensitivity testing on day 5 post SNL. KN93 (30 and 45 nmol) significantly reversed SNL-induced mechanical allodynia and thermal hyperalgesia. KN93 (15 nmol) or KN92 (45 nmol) didn't change the level of mechanical allodynia or thermal hyperalgesia. Data are expressed in Mean ± SEM. ***, $p < 0.001$, compared with the sham group; ###, $p < 0.001$, compared with the SNL group, n = 8 for each group. Arrows indicate the approximate time when KN93, KN92, or saline was injected.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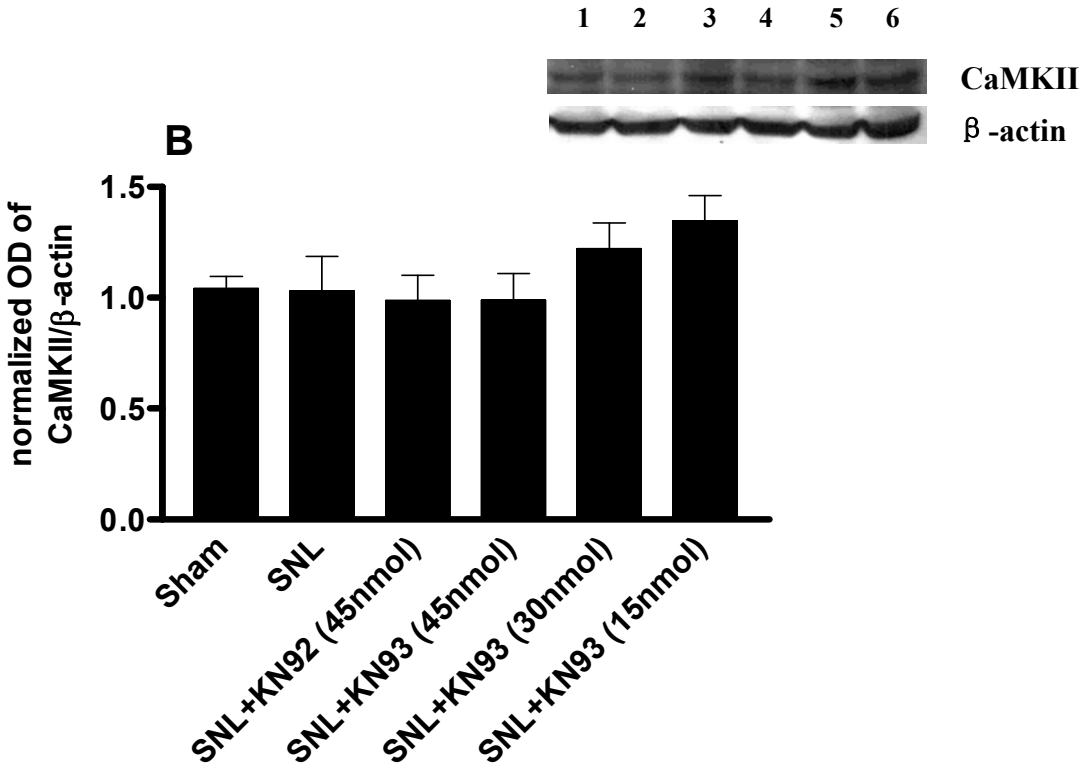
β -actin
A
normalized OD of pCaMKII/β-actin
6
4
2
0
***
***
###
###
*
Sham
SNL
SNL+KN92 (45nmol)
SNL+KN93 (45nmol)
SNL+KN93 (30nmol)
SNL+KN93 (15nmol)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β -actin
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1.0
0.5
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SNL+KN93 (45nmol)
SNL+KN93 (30nmol)
SNL+KN93 (15nmol)

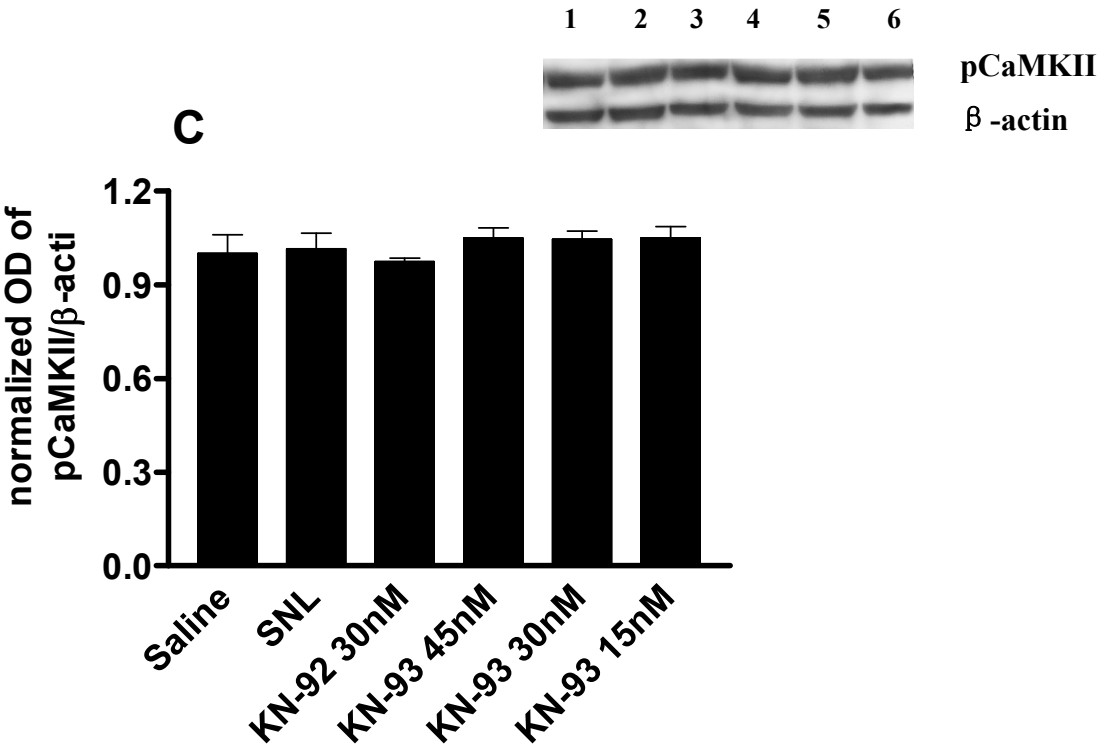


Figure 2. Suppression of spinal nerve ligation-induced spinal CaMKII activity by KN93. Ipsilateral (A) and contralateral (C) CaMKII activity, and ipsilateral CaMKII expression (B) in the lumbar sections of the spinal cord were determined by western blotting. L5/L6 spinal nerve ligation (SNL) significantly increased CaMKII activity (pCaMKII) on the ipsilateral side, but not on the contralateral side. Nor was CaMKII expression altered significantly. Acute treatment (*i.t.*) with KN93 (45 nmol, but not 30 or 15 nmol) reduced this SNL-induced spinal CaMKII activity. KN92 (45 nmol) didn't change CaMKII activity. Histogram data, expressed in Mean ± SEM, were constructed from the representative figure shown and three other experiments. *, $p < 0.05$, ***, $p < 0.001$ compared with the sham group; ###, $p < 0.001$ compared with the SNL group, n = 8 for each group.

SNL-induced activation of CaMKII (pCaMKII) (Fig. 2A, p <0.001 compared with the SNL group, n = 4). KN93 at the lowest dose used (15 nmol) did not alter pCaMKII expression, and KN92 did not affect the expression of pCaMKII (Fig. 2A, p > 0.05 compared with the SNL group, n = 4). In addition, KN93 or KN92 did not alter thermal and mechanical nociception baselines in naive mice (Luo et al., 2008). These data suggest that acute inhibition of CaMKII by KN93 dose-dependently reversed SNL-induced thermal hyperalgesia and mechanical allodynia, consistent with the inhibitor's action on CaMKII activity.

The onset and duration of action of KN93 were investigated in another series of experiments to monitor the thermal and mechanical sensitivities for up to 24 h after administration of KN93 (intrathecal injection). The antihyperalgesic/antiallodynic effect of KN93 (30 and 45 nmol) started at 30 min and peaked at 2 h (Fig. 3). At the highest dose, KN93 (45 nmol) completely reversed thermal hyperalgesia and tactile allodynia (p > 0.05 compared with the sham group at 2 h), and its action lasted for at least 4 h (Fig. 3). At a lower dose, KN93 (30 nmol) completely blocked mechanical allodynia and partially suppressed thermal hyperalgesia at the peak effect time (2 h); the antiallodynic and antihyperalgesic actions lasted for 2 and 4 h, respectively (Fig. 3). KN93 at 15 nmol did not affect either thermal or mechanical sensitivity at any time point tested. At 8 h after administration of KN93, the antihyperalgesic/antiallodynic action of KN93 had disappeared. All SNL-operated mice, either treated or not treated with KN93, had the same thermal and mechanical sensitivities on the following day (24 h) (p > 0.05 compared with the SNL group; p < 0.001 compared with the sham group,

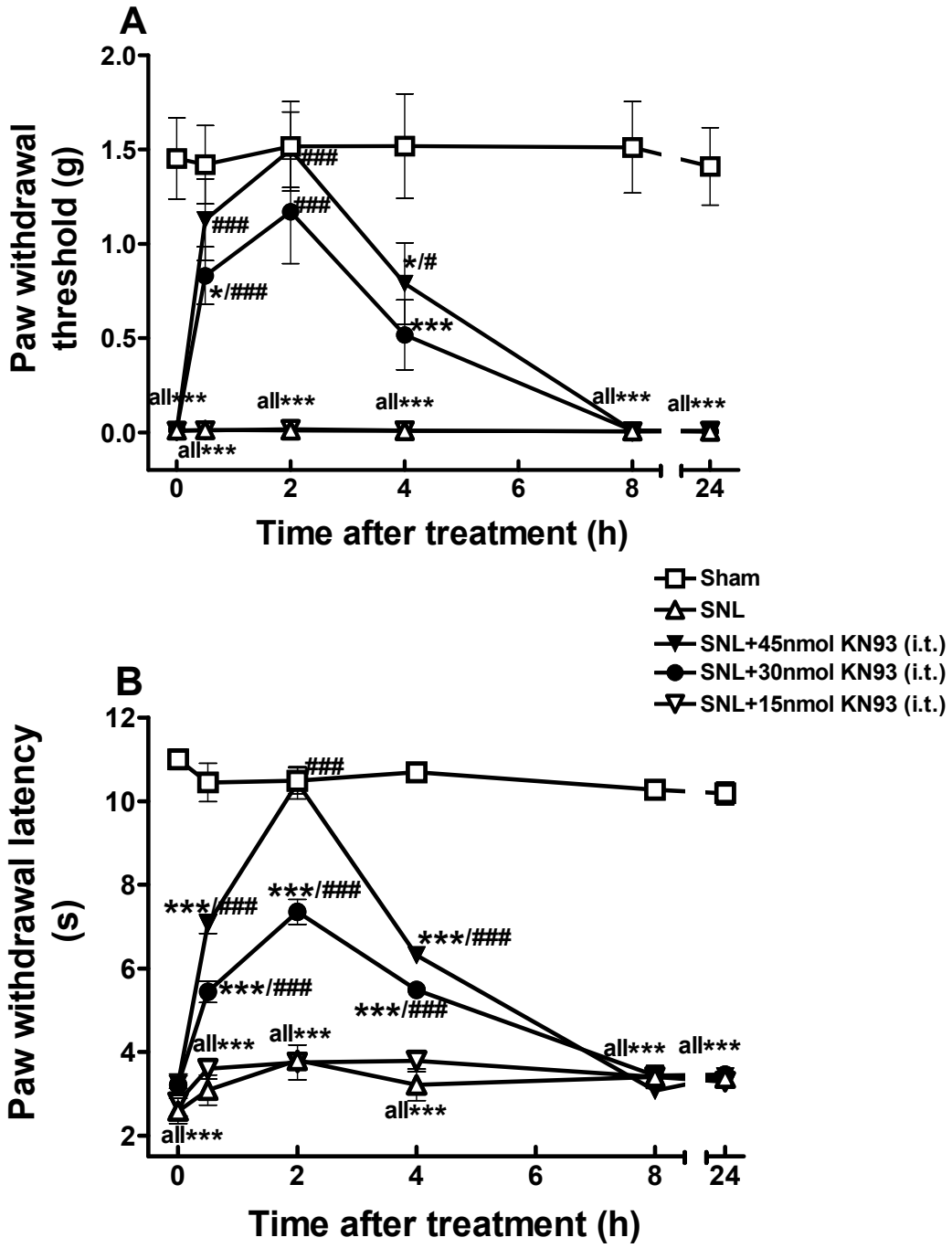


Figure 3. Time course of reversing spinal nerve ligation-induced mechanical allodynia (A) and thermal hyperalgesia (B) by KN93. Mice were administered (*i.t.*) 15-45 nmol KN93 or saline. Mechanical allodynia and thermal hyperalgesia were tested at 0, 0.5, 2, 4, 8 and 24h after the KN93 or saline injection. KN93 reversed the established spinal nerve ligation (SNL)-induced mechanical allodynia and thermal hyperalgesia in a dose and time-dependent manner. Data are expressed in Mean ± SEM. *, $p < 0.05$, ***, $p < 0.001$, compared with the sham group; #, $p < 0.05$, ###, $p < 0.001$, compared with the SNL group, n = 8 for each group.

n = 8). At the peak-effect time (2 h), ED_{50} values were estimated to be 26.1 ± 0.3 (antiallodynia) and 29.7 ± 0.1 nmol (antihyperalgesia).

3.2 Trifluoperazine Reversed SNL-Induced Hyperalgesia and Allodynia

Trifluoperazine is a clinically used antipsychotic drug that we recently discovered to inhibit CaMKII in vitro and in vivo (Tang et al., 2006c; Luo et al., 2008). Therefore, this drug would not only provide another test of our hypothesis but also might become a potential drug therapy targeting the CaMKII signaling pathway. Five days after SNL, when thermal hyperalgesia and mechanical allodynia fully developed, trifluoperazine (0.1 - 0.5 mg/kg i.p.) was administrated to determine whether the drug produced antihyperalgesic and antiallodynic actions in the SNL model of neuropathic pain. Trifluoperazine (0.5 and 0.25 mg/kg i.p.) completely reversed the established SNL-induced mechanical allodynia (Fig. 4A) and thermal hyperalgesia (Fig. 4B) ($p < 0.001$ compared with the SNL group, $n = 8$) within 2 h, whereas at a lower dose (0.1 mg/kg i.p.), it had no effect on SNL-induced hyperalgesia or allodynia (Fig. 4). Therefore, trifluoperazine was able to dose-dependently reverse SNL-induced neuropathic pain behaviors. Correlating with its behavioral effect, trifluoperazine was found to suppress SNL-induced increase of spinal CaMKII activation (pCaMKII) on the ipsilateral side in a dose-dependent manner in mice (Fig. 5A) without significantly altering the total spinal CaMKII expression (Fig. 5B) or spinal pCaMKII at the contralateral side (Fig. 5C).

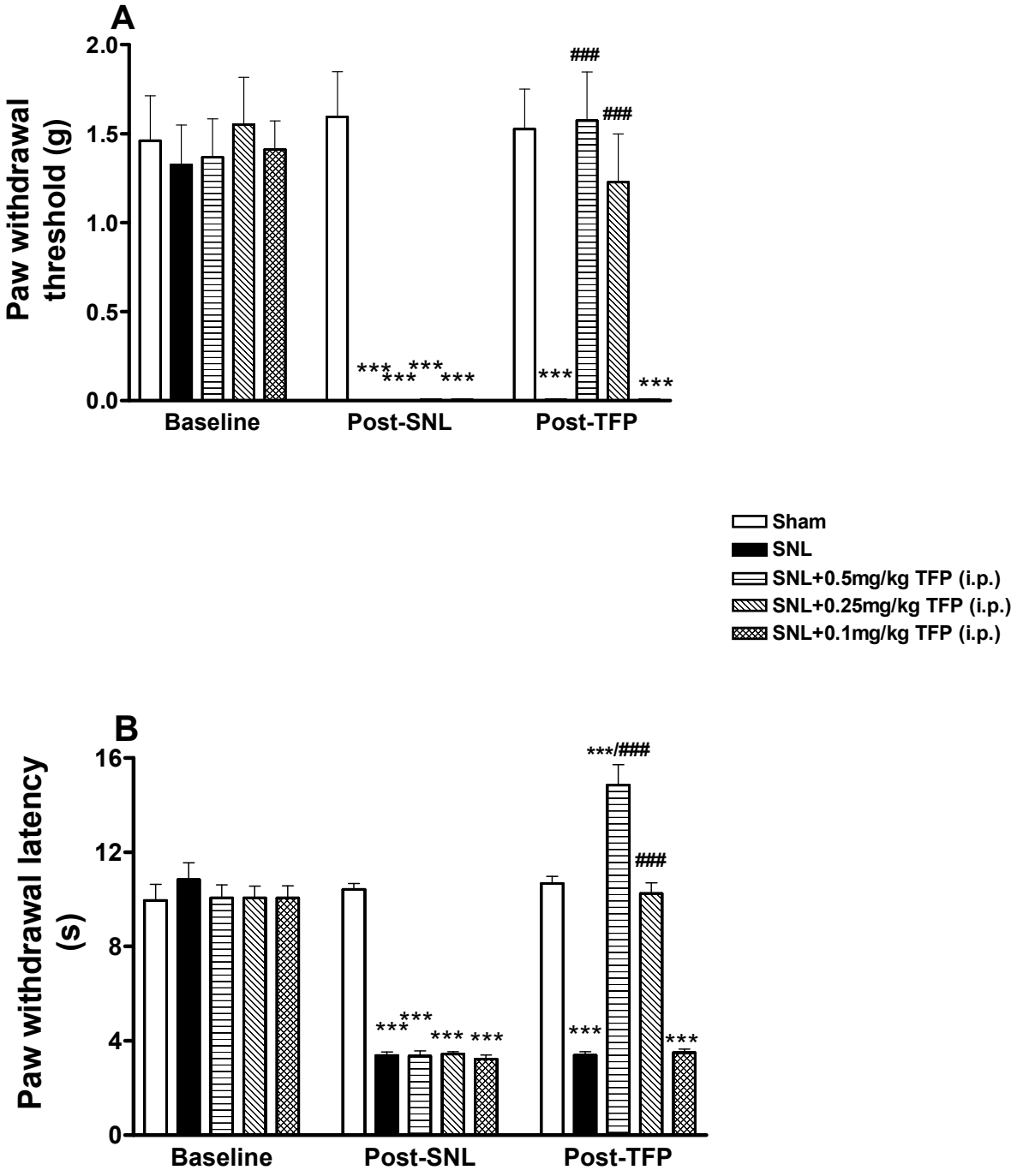
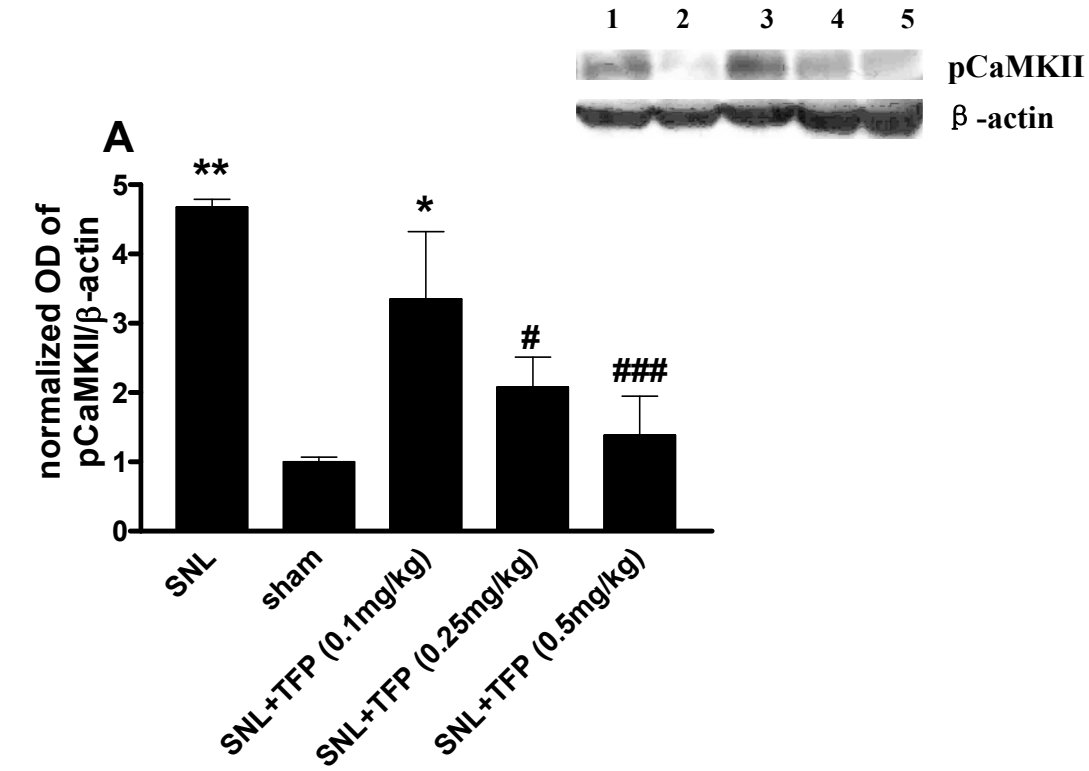


Figure 4. Reversal of spinal nerve ligation-induced mechanical allodynia (A) and thermal hyperalgesia (B) by trifluoperazine. After mechanical allodynia and thermal hyperalgesia were established 5 days post the L5/L6 spinal nerve ligation (SNL) mice, mice were treated (i.p.) with trifluoperazine (0.1-0.5 mg/kg) or saline 2h before mechanical and thermal sensitivity tests. Trifluoperazine at 0.25-0.5 mg/kg, but not 0.1 mg/kg, reversed the established SNL-induced mechanical allodynia and thermal hyperalgesia. Data are expressed in Mean ± SEM. ***, $p < 0.001$, compared with the sham group; ###, $p < 0.001$ compared with the SNL group, $n = 8$ for each group.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pCaMKII
β -actin
A
normalized OD of pCaMKII/β-actin
0
1
2
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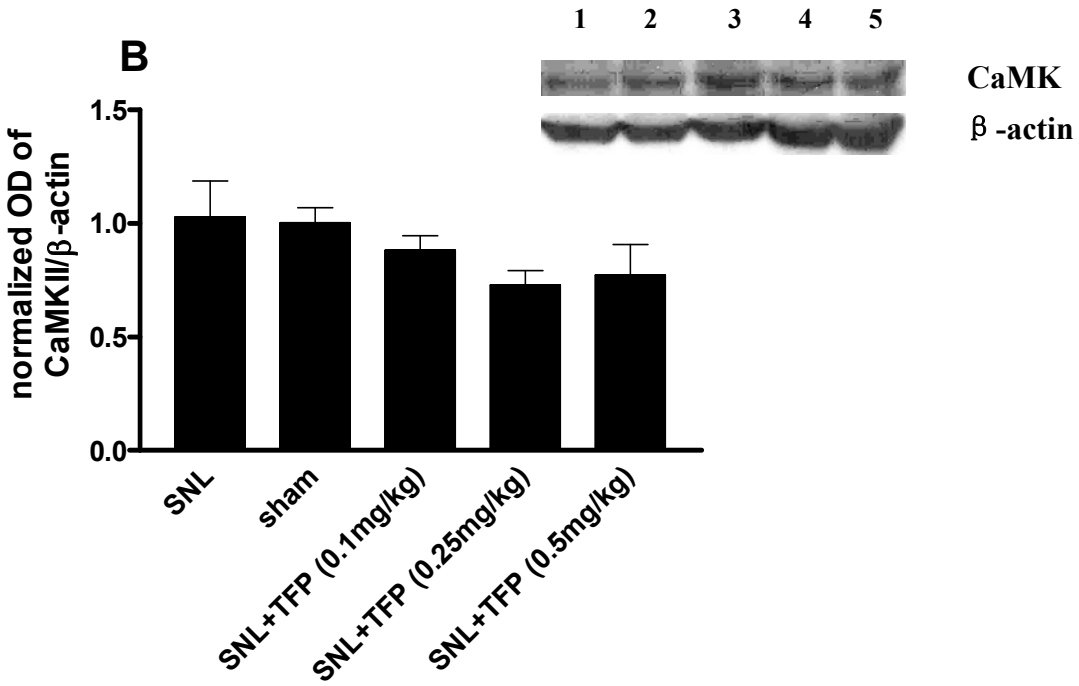
SNL
sham
SNL+TFP (0.1mg/kg)
SNL+TFP (0.25mg/kg)
SNL+TFP (0.5mg/kg)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β -actin
B
normalized OD of CaMKII/β-actin
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0.5
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SNL+TFP (0.1mg/kg)
SNL+TFP (0.25mg/kg)
SNL+TFP (0.5mg/kg)

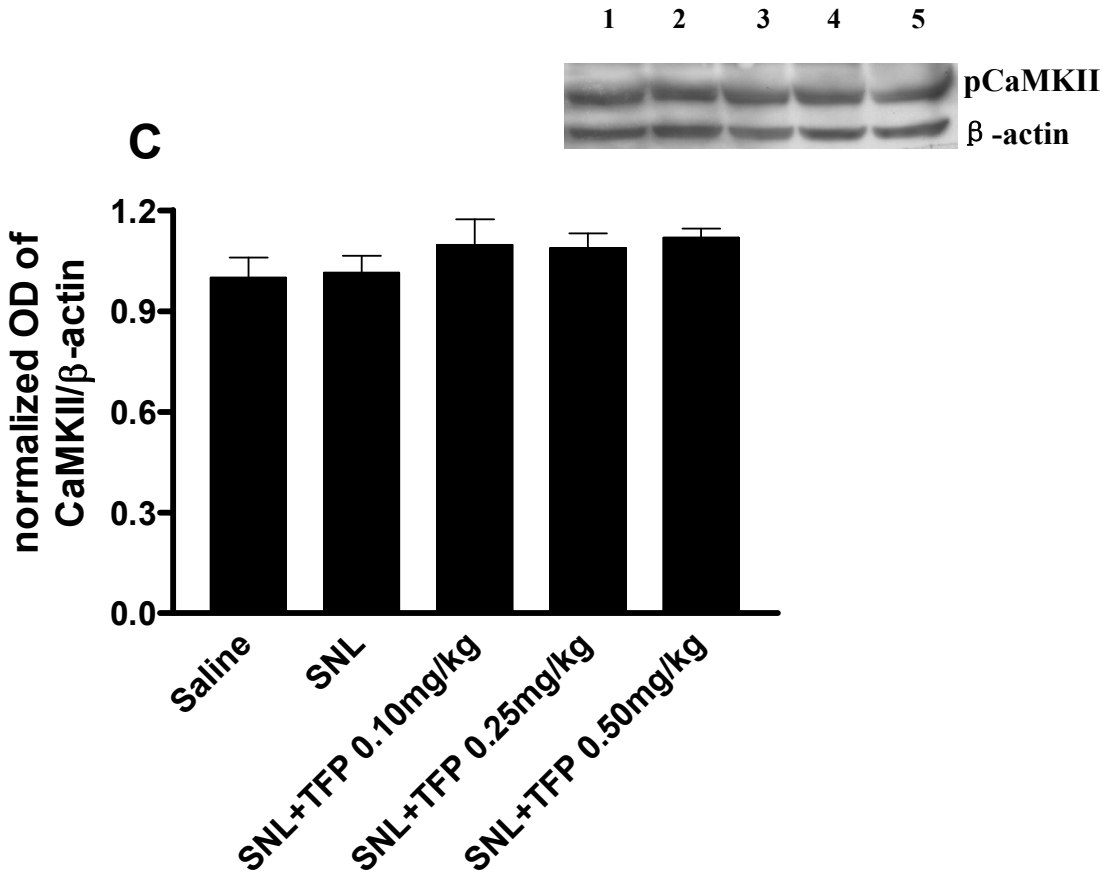


Figure 5. Suppression of spinal nerve ligation-induced spinal CaMKII activity on the ipsilateral side by trifluoperazine. The Western blotting method was used to detected ipsilateral (A) and contralateral (C) CaMKII activity, and ipsilateral CaMKII expression (B) in the lumbar sections of the spinal cord. L5/L6 spinal nerve ligation (SNL) significantly increased CaMKII activity (pCaMKII) in the lumbar spinal cord on the ipsilateral, but not contralateral side when compared with the sham group. CaMKII expression was not significantly altered by SNL or KN93. Acute treatment (*i.p.*) with trifluoperazine (0.25-0.5 mg/kg, but not 0.1 mg/kg) reduced SNL-induced spinal CaMKII activity. Histogram data, expressed in Mean ± SEM, were constructed from the representative figure shown and three other experiments. *, $p < 0.05$, ***, $p < 0.001$ compared with the sham group; #, $p < 0.05$, ###, $p < 0.001$ compared with the SNL group, n = 8 for each group.

At the highest dose, trifluoperazine (0.5 mg/kg) appeared to produce analgesia in the radiant heat assay (Fig. 4B). To better understand the nature of the drug's antihyperalgesic/antiallodynic and possible analgesic actions, we monitored its actions for 24 h after an acute intraperitoneal administration in the SNL- or sham-operated mice (Fig. 6). In the mechanical sensitivity study, trifluoperazine (0.25 - 0.5 mg/kg) exhibited a rapid onset of action in reversing the established SNL-induced mechanical allodynia. The antiallodynic effect started at 30 min and peaked at 4 h (Fig. 6A). At 4 h, the ED_{50} was estimated to be 0.2 ± 0.0 mg/kg. The antihyperalgesic effect peaked at 2 h (EC_{50}, 0.2 ± 0.0 mg/kg), and lasted for at least 4 h (for a 0.25 mg/kg dose) to 8 h (for a 0.5 mg/kg dose) (Fig. 6B). We did not find any significant action of trifluoperazine at 0.1 mg/kg on SNL-induced thermal hyperalgesia or mechanical allodynia (Figs. 4 and 6). On the next day (24 h after trifluoperazine), all SNL-operated groups showed thermal hyperalgesia and mechanical allodynia. These data suggest that trifluoperazine is able to acutely reverse the established SNL-induced abnormal pain behaviors with a duration of action of approximately 4 to 8 h. Only the highest dose (0.5 mg/kg) produced analgesic action in the radiant heat assay at 2 h.

Because trifluoperazine can be taken orally, we further examined whether oral trifluoperazine (via gastric gavage) was effective. Five days after the SNL operation, when thermal hyperalgesia and mechanical allodynia have fully developed, trifluoperazine dose-dependently attenuated SNL-induced mechanical allodynia (Fig. 7A) and thermal hyperalgesia (Fig. 7B) within 2 h after an oral administration (0.1 - 1 mg/kg). At the two higher doses, the drug completely (1 mg/kg) or partially (0.3 mg/kg)

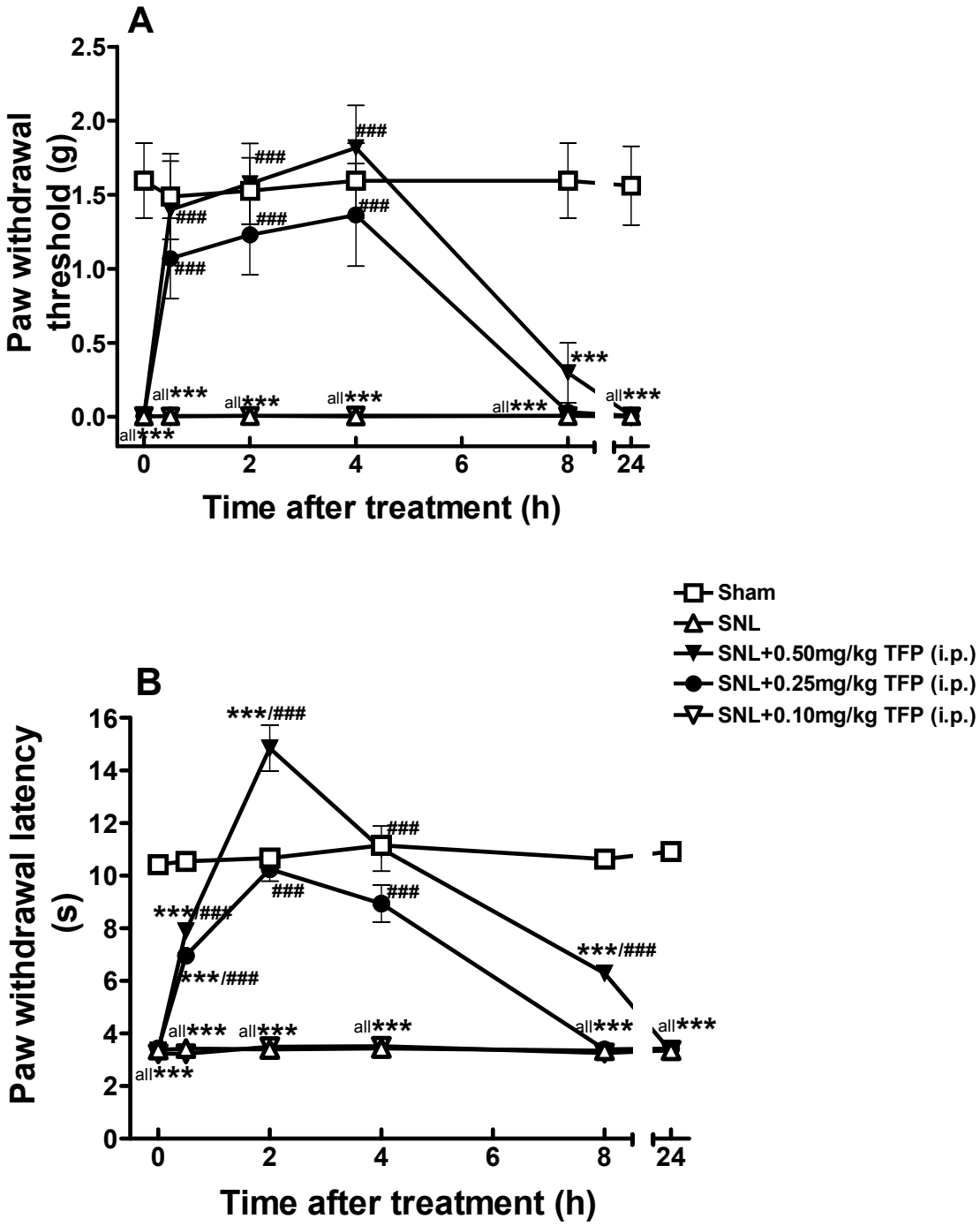


Figure 6. Time course of reversing spinal nerve ligation-induced mechanical allodynia (A) and thermal hyperalgesia (B) by trifluoperazine (*i.p.*). Mice were administered (*i.p.*) with trifluoperazine (0.1-0.5 mg/kg) or normal saline. Mechanical allodynia and thermal hyperalgesia were tested at 0, 0.5, 2, 4, 8 and 24h after the trifluoperazine or saline injection. Trifluoperazine dose- and time-dependently reversed established SNL-induced mechanical allodynia and thermal hyperalgesia. Data are expressed in Mean ± SEM. ***, $p < 0.001$, compared with the sham group; ###, $p < 0.001$, compared with the SNL group, n = 8 for each group.

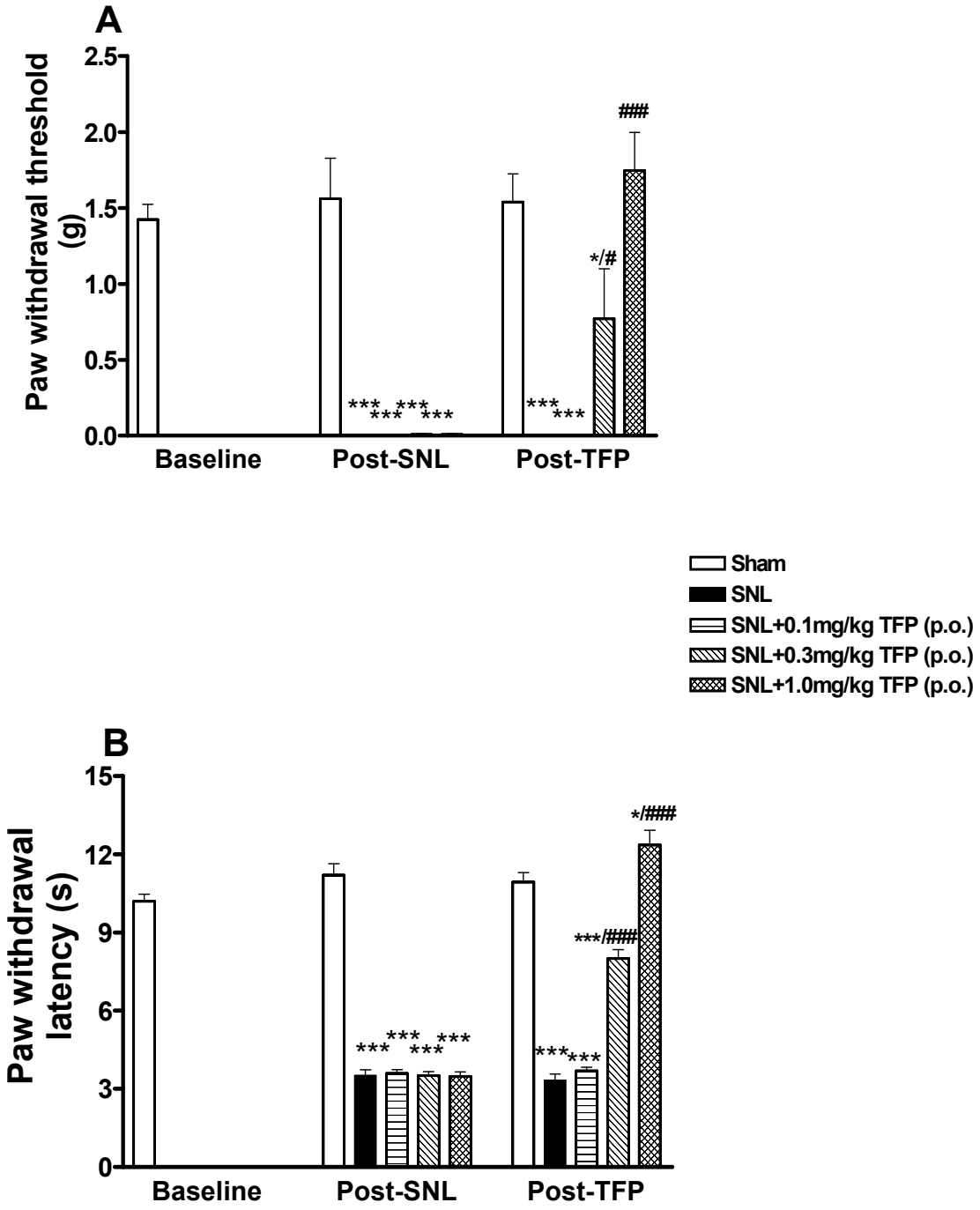


Figure 7. Reversal of spinal nerve ligation-induced mechanical allodynia (A) and thermal hyperalgesia (B) by orally administered trifluoperazine. Mice were treated with trifluoperazine (0.1-1.0 mg/kg, gastric gavage) or saline 2h before the thermal and mechanical sensitivity tests. Trifluoperazine at 0.3-1.0 mg/kg, but not 0.1 mg/kg reversed the established SNL-induced mechanical allodynia and thermal hyperalgesia. Data are expressed in Mean ± SEM. ***, $p < 0.001$, compared with the sham group; ###, $p < 0.001$ compared with the SNL group, n = 8 for each group.

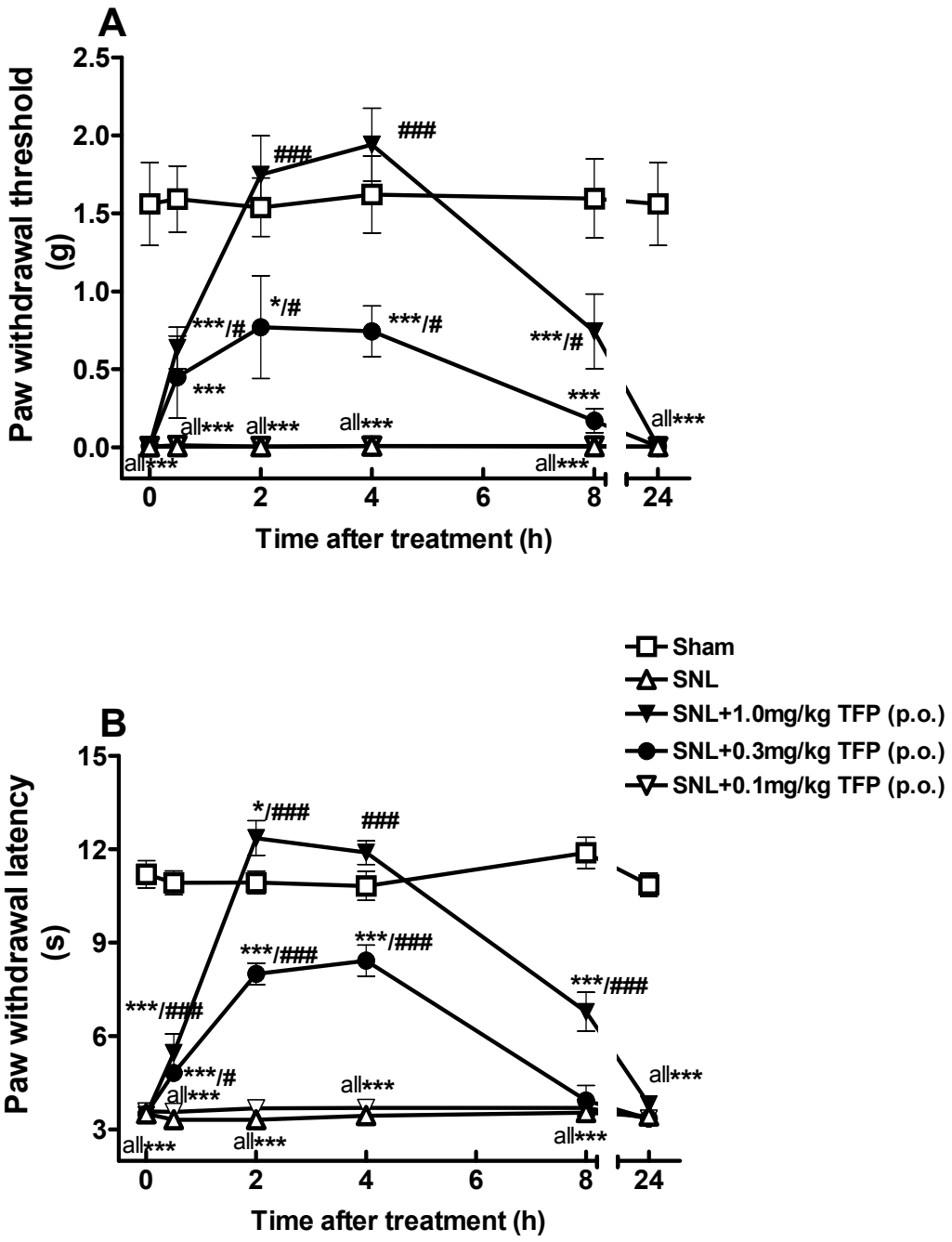


Figure 8. Time course of reversing spinal nerve ligation-induced mechanical allodynia (A) and thermal hyperalgesia (B) by orally administered trifluoperazine. Mice were administered with trifluoperazine (0.1-1.0 mg/kg, gastric gavage) or normal saline. Mechanical allodynia and thermal hyperalgesia were tested at 0, 0.5, 2, 4, 8 and 24h after the trifluoperazine or saline injection. Trifluoperazine reversed the established SNL-induced mechanical allodynia and thermal hyperalgesia in a dose- and time-dependent manner. Data are expressed in Mean ± SEM. *, $p < 0.05$, ***, $p < 0.001$, compared with the sham group; #, $p < 0.05$, ###, $p < 0.001$, compared with the SNL group, n = 8 for each group.

or 1.0 mg/kg p.o.) or equal volumes of saline (administered intraperitoneally or by mouth). Four hours later when the drug's antihyperalgesic/antiallodynic actions were at the peak, locomotor activity was examined. Trifluoperazine did not cause locomotor impairment in mice ($p > 0.05$ compared with the corresponding saline group, $n = 5$; Table 1).

4. Discussion

In this study, we examined the role of CaMKII in an experimental model of neuropathic pain. Our findings supported the hypothesis that CaMKII is required for the maintenance of neuropathic pain behaviors induced by spinal nerve ligation. Such a mechanism is plausible considering that, in the primary afferent and the spinal cord, CaMKIIα is specifically expressed in the small- to medium-diameter primary sensory neurons in the dorsal root ganglia and in the superficial laminae of the spinal cord dorsal horn. Both locations are important for the transmission and processing of nociceptive signals (Bruggemann et al., 2000; Carlton, 2002).

In the formalin inflammation model, the second phase of formalin-induced paw-licking behavior was significantly reduced by KN93 (Choi et al., 2006) and largely absent in CaMKIIα(T286A) mutant mice (Zeitz et al., 2004). We found that KN93, but not its inactive analog KN92, prevented or reversed CFA-induced mechanical allodynia and thermal hyperalgesia (Luo et al., 2008). Our data are in agreement with the findings from the persistent-inflammation model that CaMKII is critical for the presence of persistent pain. Our findings, however, are contrary to the results of a

Table 1. Effect of trifluoperazine on locomotor ability

Mice received trifluoperazine (0.5 mg/kg i.p. or 1.0 mg/kg p.o.) or equal volumes of normal saline (intraperitoneally or by mouth) 4 h before the rotarod test. The duration, up to 300 s, that mice remained on the rotarod revolving at 16 rpm was recorded. Trifluoperazine at the doses and time that showed peak antiallodynic/antihyperalgesic effects did not affect the locomotor activity in mice. Data are expressed in mean ± S.E.M., *n* = 5 for each group. No statistical differences were identified.

	Intraperitoneal Administration		Oral Administration	
	Saline	TFP (0.5 mg/kg)	Saline	TFP (1.0 mg/kg)
Duration on rotarod (s)	218.0 ± 107.3	192.6 ± 65.3	235.4 ± 110.3	213.2 ± 51.8

TFP: trifluoperazine.

previous study where KN93 was ineffective in reversing fully developed thermal hyperalgesia and mechanical allodynia 7 days after CCI (Dai et al., 2005). The discrepancy may be due to the different pain models that were used. However, our previous studies have suggested that different pain states may have different degrees of CaMKII activity and, therefore, may require different doses of CaMKII inhibitors (Luo et al., 2008). We were able to confirm the inhibition of CaMKII by KN93 or trifluoperazine at the doses that produced behavioral effects, but not at lower doses where neither significant CaMKII inhibition nor behavioral effects occurred. Another previous study had found that KN93 (120 pmol i.t.) and myristoyl-autocamtide 2-related inhibitory peptide (1 nmol i.t.) effectively reversed CCI-induced pain behaviors in mice, although experimental details were not provided (Garry et al., 2003). Although the overall outcome of the study by Garry et al. (2003) agreed with outcome of the current study, the doses of KN93 used varied significantly. We found that KN93 at 15 nmol i.t. or lower was ineffective at any time point after drug administration (Fig. 3).

Findings from our laboratory and others (Garry et al., 2003; Dai et al., 2005; Ogawa et al., 2005; Luo et al., 2008) have so far suggested a critical role of CaMKII in persistent pain. This was not supported by a study using CaMKIIα(T286A) mutant mice (Zeitz et al., 2004). CaMKIIα(T286A) mutant mice express a form of CaMKIIα that cannot be autophosphorylated. Therefore, CaMKII remains inactive. The second phase of formalin-induced paw-licking behavior was largely absent in CaMKIIα(T286A) mice. However, nerve injury and CFA produced similar enhancement of thermal and

mechanical sensitivity in CaMKIIα mutant and wild-type mice (Zeitz et al., 2004). The exact cause for the discrepancy is not clear. Obviously, two different approaches were used in these studies by either chemically inhibiting CaMKII or genetically eliminating functional CaMKII. Interpreting studies using CaMKII inhibitors can be complicated by the possible loss of selectivity, especially at high doses. However, mouse transgenic/"knockout" approaches may have potential problems with compensatory changes, unmatched genetic background, or other nonspecific genomic effects.

In our studies, the effect of acute CaMKII inhibition in reversing the established persistent pain was further supported by experiments employing trifluoperazine. Trifluoperazine is a clinically used antipsychotic drug and a calmodulin inhibitor that suppresses CaMKII activity (Tang et al., 2006c; Luo et al., 2008). Therefore, trifluoperazine not only presents a unique opportunity to test our hypothesis but also may provide a clinically useful drug for alleviating neuropathic pain by targeting the CaMKII pathway. Indeed, systemic trifluoperazine (intraperitoneally or by mouth) dose dependently reversed mechanical allodynia and thermal hyperalgesia in SNL-operated mice. Therefore, we have used two very different drugs that converge on the same CaMKII mechanism that was confirmed by biochemical analysis. These data strongly indicate that KN93 and trifluoperazine exhibited their antihyperalgesic/antiallodynic actions through CaMKII. It is unlikely that another mechanism could have accounted for the action of trifluoperazine and KN93 (controlled by KN92). On the other hand, we can not exclude such a possibility.

Our data suggest that trifluoperazine may become useful for the treatment of

neuropathic pain, although it has yet to be demonstrated in clinical settings. The side effects associated with the drug, including dystonia, hyperprolactinemia, akathisia, sedation, hypotension, confusion, dry mouth, blurred vision, and urinary retention, may become a problem (Marques et al., 2004). These side effects are probably caused by the drug's pharmacologic actions of blocking the dopamine receptors and, to a lesser degree, antagonizing the cholinergic, histaminic, and α-adrenergic receptors (Marques et al., 2004). However, the antiallodynic/antihyperalgesic doses of trifluoperazine, with ED_{50} of 0.2 mg/kg i.p. to 0.3 mg/kg p.o., are only one tenth of the antipsychotic doses. Trifluoperazine exhibited similar potency in preventing or reversing CFA-induced persistent pain behaviors (Luo et al., 2008). The antipsychotic effect ED_{50} of the drug has been reported to be approximately 3 to 5 mg/kg (Reiss et al., 1994). Moreover, at the antihyperalgesic/antiallodynic doses (0.5 mg/kg i.p. or 1 mg/kg p.o.), trifluoperazine did not cause locomotor impairment, which was in agreement with previous findings in the open field test (DeLong et al., 1985). One previous study reported that trifluoperazine (intrathecal injection.) produced either analgesia (at low doses) or hyperalgesia (at a high dose) in a formalin-induced inflammatory pain model (Golbidi et al., 2002). In naive mice, trifluoperazine (0.5 mg/kg i.p.) produced a weak but significant thermal hyperalgesic action 8 h after the drug administration; no mechanical hyperalgesia was observed (Luo et al., 2008). We did not observe hyperalgesia activity in the SNL-treated mice. Instead, trifluoperazine at the highest dose given (0.5 mg/kg i.p. or 1 mg/kg p.o.) produced an analgesic action in the thermal (but not mechanical) nociception test, 2 h after administration, on top of its

antihyperalgesic and antiallodynic effects. The SNL-induced CaMKII activation occurred only in the ipsilateral but not the contralateral spinal cord. Similar unilateral activation of CaMKII was found after CCI operation in rats (Dai et al., 2005).

Although the downstream effect of CaMKII in leading to persistent pain is not entirely clear, its interaction with the NMDA receptors may provide a plausible mechanism. There seemed to be a feed-forward interaction between the NMDA receptors and CaMKII in the persistent pain state (Luo et al., 2008). Phosphorylation of NMDA receptors by CaMKII has been shown to enhance the NMDA receptor function, leading to the influx of Ca^{2+} through the channels (Kitamura et al., 1993). Therefore, activation of CaMKII as a result of nerve injury can potentially increase the activity of the NMDA receptors leading to Ca^{2+} influx. Increased cytosolic Ca^{2+} ions bind and change the conformation of CaM, which in turn leads to the activation of more CaMKII (Strack et al., 1997). It has been demonstrated that NMDA receptor antagonists, administered before peripheral nerve injuries, suppress the onset of hyperalgesia. They also attenuated fully developed hyperalgesia when administered after the injury (e.g., (Dickenson et al., 1997; Zhang et al., 1998; Dai et al., 2005). Therefore, CaMKII may work in concert with NMDA receptors leading to the manifestation of neuropathic pain. There may also be a functional interaction between CaMKIIα and transient receptor potential vanilloid type 1 receptor. In rat trigeminal ganglion neurons, it was found that capsaicin increased CaMKIIα activity in the capsaicin receptor transient receptor potential vanilloid type 1 receptor-positive neurons (Price et al., 2005). In these trigeminal ganglion neurons, capsaicin- or *n*-arachidonoyl-dopamine-evoked

calcitonin gene-related peptide release was inhibited by KN93 (Price et al., 2005).

In summary, our findings suggest the critical involvement of CaMKII in maintaining experimental neuropathic pain. Such data are critical for designing new pain therapies, because most patients with persistent abnormal pain seek medical treatment after nerve or tissue injuries. To begin to translate these findings to clinically useful drugs, we further found that trifluoperazine at relatively low doses (approximately one-tenth of its antipsychotic doses) could effectively block SNL-induced abnormal pain behaviors. Although the drug is not expected to be selective for CaMKII and has side effects, it is orally available and has been used in humans for more than 30 years. Therefore, trifluoperazine may provide a new drug candidate for the treatment of neuropathic pain by targeting the CaMKII pathways.

IV. Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE IIα IS REQUIRED FOR THE INITIATION AND MAINTENANCE OF OPIOID-INDUCED HYPERALGESIA

1. Introduction

Opioids are commonly prescribed to treat chronic pain. However, repeated administration of opioids not only leads to tolerance and dependence, but also results in opioid-induced hyperalgesia (OIH), defined as a lowered pain threshold caused by opioid exposure (Vanderah et al., 2001; Mao, 2002; Ossipov et al., 2005; Chu et al., 2008). Although hyperalgesia associated with opioid use has been reported clinically for decades, the underlying mechanism of OIH remains unclear. Opioid-induced hyperalgesia is considered a unique phenomenon that can be distinguished from opioid withdrawal-induced hyperalgesia (Li et al., 2001b; Angst and Clark, 2006). When opioids are used for the treatment of underlying painful conditions, OIH may differ from the original pain condition in its quality and affected location (Chu et al., 2008). Clinically, OIH and opioid tolerance can both result in similar increased requirement of opioid dosage; however, OIH causes a downward shift of the opioid dose–response curve whereas tolerance leads to a rightward shift of the curve (Chu et al., 2008).

A growing number of reports have reproducibly demonstrated OIH in rodent models (Kayan et al., 1971; Gardell et al., 2002; Liang et al., 2006). Although a short onset (within 2 - 3 h) of mechanical allodynia and thermal hyperalgesia has been

reported after acute administration of opioids, such as fentanyl and heroin (Celerier et al., 1999; Celerier et al., 2000), chronic morphine administration with repeated intermittent injections or subcutaneous pellet implantation produced prolonged and robust OIH (Li et al., 2001b; Liang et al., 2008).

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine protein kinase that is colocalized with the μ-opioid receptor in the superficial laminae of the spinal dorsal horn and in the small to medium diameter primary afferent neurons in the dorsal root ganglion (Bruggemann et al., 2000; Carlton, 2002). Activation of CaMKII requires elevated intracellular Ca^{2+} and activated calmodulin. Supraspinal or spinal inhibition of CaMKII has been shown to prevent or reverse morphine tolerance and dependence (Tang et al., 2006a). Moreover, spinal CaMKIIα was activated in capsaicin-induced inflammation (Fang et al., 2002), complete Freund's adjuvant (CFA)-induced inflammatory pain (Luo et al., 2008), and spinal nerve ligation (SNL)-induced neuropathic pain states (Chen et al., 2009). CFA- or SNL-induced thermal hyperalgesia and mechanical allodynia can be dose dependently reversed by CaMKII inhibitors (Luo et al., 2008; Chen et al., 2009). A role for CaMKII in OIH has not been reported. In this study, we tested the hypothesis that CaMKIIα is a critical cellular signaling mechanism leading to opioid induced hyperalgesia. We first used KN93, the most commonly used CaMKII inhibitor, and its kinase-inactive chemical analog KN92 in the study (Sumi et al., 1991). To overcome potential nonspecific actions of KN93 and to investigate the specific CaMKII isoform involved, we also used small interfering RNA (siRNA) to knock down CaMKIIα.

Furthermore, the hypothesis was tested in mice lacking the CaMKIIα autophosphorylation site.

2. Materials and Methods

2.1 Materials

Morphine sulfate (MS), morphine, and placebo pellets were obtained from the National Institute on Drug Abuse (Rockville, MD). 2-[*N*-(2-Hydroxyethyl)-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine) (KN93) and 2-[*N*-(4-methoxybenzenesulfonyl)] amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzyl-amine (KN92) were purchased from Calbiochem. Other chemicals were purchased from Sigma.

2.2 Animals

Male ICR mice (20–25 g, Harlan Laboratories) were kept under standard conditions with a 14/10 h light/dark cycle (5:00 A.M. to 7:00 P.M.) and allowed food and water ad libitum before experimental procedures. For CaMKIIαT286A mutant mice, breeders were generously provided by Dr. Alcino Silva, University of California Los Angeles (Giese et al., 1998). These mice were back-crossed with C57BL/6J mice for 10 generations. Heterozygous breeding was used to generate male homozygous mutant mice and littermate wild-type control mice for this study. Both genotypes were viable and showed normal growth and reproduction. Genotyping of litters was performed by PCR using a set of primers (5'-CTGTACCAGCAGATCAAAGC-3', 5'-ATCACTAGCACCATGTGGTC-3'). The PCR products for wild-type and mutant

alleles are 200 and 290 bp, respectively. Each experimental mouse was genotyped twice using DNA from two separate extractions from the tail tissue samples. The investigator who performed the biochemical and behavioral tests was blind to mouse genotypes. All breeding and experiments were performed in accordance with the policies and recommendations of the International Association for the Study of Pain (IASP) and the NIH guidelines after approval by the University of Illinois Institutional Animal Care and Use Committee.

2.3 Methods

2.3.1 OIH induced by repeated subcutaneous administration

To induce OIH, mice were treated subcutaneously according to a previously published schedule (Liang et al., 2006). Mice received 20 mg/kg morphine sulfate (twice per day, s.c.) for 3 consecutive days and two more injections of 40 mg/kg morphine sulfate on day 4. Control mice received equal volume and number of saline injections (subcutaneous). Mechanical and thermal sensitivities were tested before and after morphine treatment.

2.3.2 OIH induced by morphine pellet implantation

Separate groups of 5 male ICR mice were implanted subcutaneously with morphine pellets or placebo pellets (1 pellet/mouse, each morphine pellet contains 75 mg of morphine base; a placebo pellet is contains no morphine). This treatment not only induces opioid dependence and tolerance (Tang et al., 2006a), but also OIH ((Li

et al., 2001a). To determine the presence of OIH, mechanical and thermal sensitivities were tested before and after morphine treatment as described below.

2.3.3 Drug and siRNA administration

KN93 and KN92 were administered intrathecally by percutaneous puncture through the L5-L6 intervertebral space, as described previously (Hylden and Wilcox, 1980; Chen et al., 2009). A lateral tail flick was considered as success of the intrathecal injection. To inhibit CaMKIIα, CaMKIIα was targeted by small interfering RNA (siRNA). Four days after morphine pellet implantation, mice were treated with CaMKIIα siRNA (5'-CACCACCAUUGAGGACGAAdTdT-3', 3'-dTdTGUGGUGG UAACUCCUGCUU-5') (Zayzafoon et al., 2005) or Stealth RNAi negative control (Invitrogen) (2 μg, i.t., twice per day for 3 consecutive days). These oligos were mixed with the transfection reagent i-Fect (Neuromics), in a ratio of 1:5 (w/v) (Luo et al., 2005). Mechanical and thermal sensitivity tests were performed daily.

2.3.4 Mechanical Allodynia

Mechanical sensitivity was assayed using calibrated von Frey filaments (Stoelting) according to the “up-down” paradigm (Luo et al., 2008; Chen et al., 2009). Briefly, mice were placed on wire mesh platforms in individual Plexiglas containers. After 30 min of acclimation, each von Frey filament was pressed upward to the midplantar surface for 5 s or until a withdrawal response occurred. Withdrawal of the hindpaw was scored as a response. The up-down algorithm was used to determine

50% probability of paw withdrawal threshold (Luo et al., 2008).

2.3.5 Thermal Hyperalgesia

Thermal sensitivity was measured using a plantar tester (model 7372, UGO BASILE) as described previously (Hargreaves et al., 1988; Wang et al., 2001; Chen et al., 2009). In these experiments, mice were placed in a clear plastic enclosure with a glass floor. After a 30 min period of habituation, radiant heat stimulation was applied to the middle portion of the planter surface of the hindpaw, and paw withdrawal latencies were measured. The heat intensity was adjusted to pro-duce a baseline response around 10 s. To prevent tissue damage, a cutoff time of 20 s was applied.

2.3.6 Rotarod test

To exclude the possibility that KN93 may cause locomotor impairment, a rotarod test was performed as described previously (Prestori et al., 2008). Mice were trained to remain on a fixed speed (4 rpm) rotarod for 60 s (model series 8; IITC Life Science). On the following day, mice were retrained and those that failed to stay on the rotarod for 60 s were not used in further studies. Baseline was tested 30 min later by placing the mice on an accelerating rotarod (4 - 40 rpm over 300 s). The latency to fall off the rotarod was recorded. Mice were then treated intrathecally with either KN93 (45 nmol), KN92 (45 nmol), or saline and retested 0.5, 1, 2, 4, and 8 h later. The cutoff was set at 300 s.

2.3.7 Immunoblotting

Mice were killed and lumbar spinal cords were quickly dissected and frozen on dry ice for Western blotting analysis, as previously described (Tang et al., 2006a; Chen et al., 2009). Briefly, tissues were homogenized in ice-cold radioimmunoprecipitation assay buffer and centrifuged. Aliquots of supernatant samples were used to determine protein content by a modified Bradford method (Pierce). Samples (60 μg of total protein) were separated by 12% SDS-PAGE and electrotransferred onto PVDF membrane. The membrane was probed with a rabbit anti-(T286)pCaMKIIα antibody (1:1000, Santa Cruz Biotechnology) or a mouse anti-CaMKIIα antibody (1:1000, Santa Cruz Biotechnology) at room temperature for 3 h, followed by incubation with HRP-conjugated donkey anti-rabbit (for pCaMKIIα) or anti-mouse (for CaMKIIα) secondary antibody (1:1000, GE Healthcare). An enhanced chemiluminescence detection system (ECL, GE Healthcare) was applied for development. The membrane was then stripped and reprobed with a mouse anti-β-actin antibody (1:10,000, Santa Cruz Biotechnology) followed by a HRP-conjugated anti-mouse secondary antibody (1:1000, GE Healthcare) and developed as above. ECL signals were detected using a ChemiDoc system and analyzed using the Quantity One program (Bio-Rad). CaMKII immunoreactivity was expressed as the ratio of the opticaldensities of pCaMKII or CaMKIIα to those of β-actin.

2.3.8 Immunohistochemistry

Immunohistochemical staining of spinal CaMKII was carried out according to the method previously described (Wang et al., 2001). Mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg, i.p.). The vascular system was perfused with 60 ml of 4°C PBS, pH 7.4, and followed by 50 ml of 2% paraformaldehyde solution. The lumbar spinal cords were removed and postfixed overnight in 4% paraformaldehyde at 4°C, and cryoprotected in 30% sucrose for 24 h at 4°C. Spinal cord sections were cut on a cryostat at 20 μm thickness and washed 2 times with cold PBS. Sections were preincubated with 0.3% H_2O_2 and blocked with 10% goat serum (Invitrogen) and 0.3% Triton X-100 in PBS for 20 min. Floated sections were incubated with the primary antibody for pCaMKIIαThr286 (1:1000) overnight at room temperature, followed by another incubation with biotinylated goat anti-rabbit IgG secondary antibody (1:200, Vector Laboratories) at room temperature for 2 h. Incubation in the absence of the first antibody or in the presence of pCaMKIIα Thr286 blocking peptide (Santa Cruz Biotechnology) was used to control for antibody specificity. The sections were developed using Elite Vectastain ABC kit (Vector Laboratories). Diaminobenzidine (DAB) stained sections were imaged by inverted microscopy (Olympus) and quantified using the Meta-Morph Imaging Software (Universal Imaging). For each condition, 5 sections and 6 areas from each section were analyzed and averaged.

2.3.9 Statistical analysis

All data are presented as mean ± SEM. Comparisons between groups were

analyzed using Student's t test (two groups) or a two-way repeated measure ANOVA followed by post hoc analyses using Dunnett's t test (multiple groups). Statistical significance was established at 95% confidence limit.

3 Results

3.1 Chronic morphine exposure-induced mechanical allodynia and thermal hyperalgesia in mice

Repeated subcutaneous morphine administration and subcutaneous morphine pellet implantation are two commonly used OIH models in mice. Four days of subcutaneous morphine administration by intermittent injections significantly increased mechanical and thermal sensitivities compared with saline-treated mice (Fig. 1A,B). The mechanical allodynia and thermal hyperalgesia were detectable on day 5 and lasted for approximately 2 weeks before recovery ($p < 0.001$ compared with saline control, $n = 5$).

Continuous morphine exposure using pellet implantation also induced OIH. Mice were implanted subcutaneously with morphine pellets (75 mg/pellet) or placebo pellet and mechanical and thermal sensitivities were measured daily for 15 d. Morphine implantation initially produced antinociception in both mechanical ($p < 0.001$, compared with the placebo group, $n = 5$) and thermal sensitivity tests ($p < 0.05$, compared with the placebo group, $n = 5$). This was followed by a decrease in paw withdrawal threshold and latency. Mechanical allodynia was developed on day 6 and lasted for 7 d ($p < 0.001$, compared with placebo group, $n = 5$) (Fig. 2A,B). Thermal

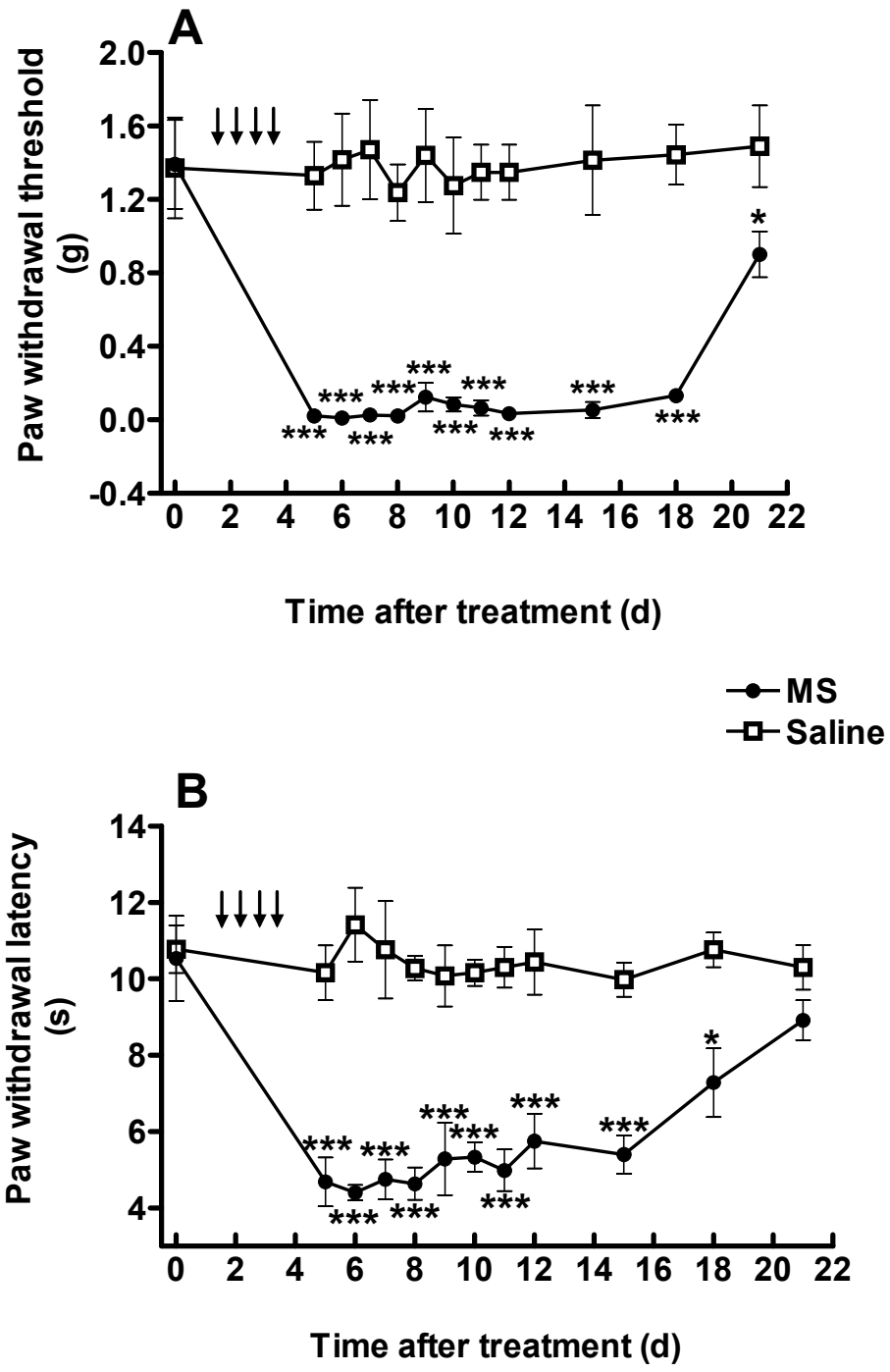


Figure 1. Repeated intermittent morphine administration induced mechanical allodynia (A) and thermal hyperalgesia (B). Mice received saline or morphine sulfate (day 1-3: 20 mg/kg; day 4: 40 mg/kg; twice daily, *s.c.*). The paw withdrawal threshold to von Frey filament probing and withdrawal latency to radiant heat were determined. Data are expressed in Mean ± SEM. *p<0.05, ** p<0.01, *** p < 0.001, compared with the saline treated group; n = 5 for each group.

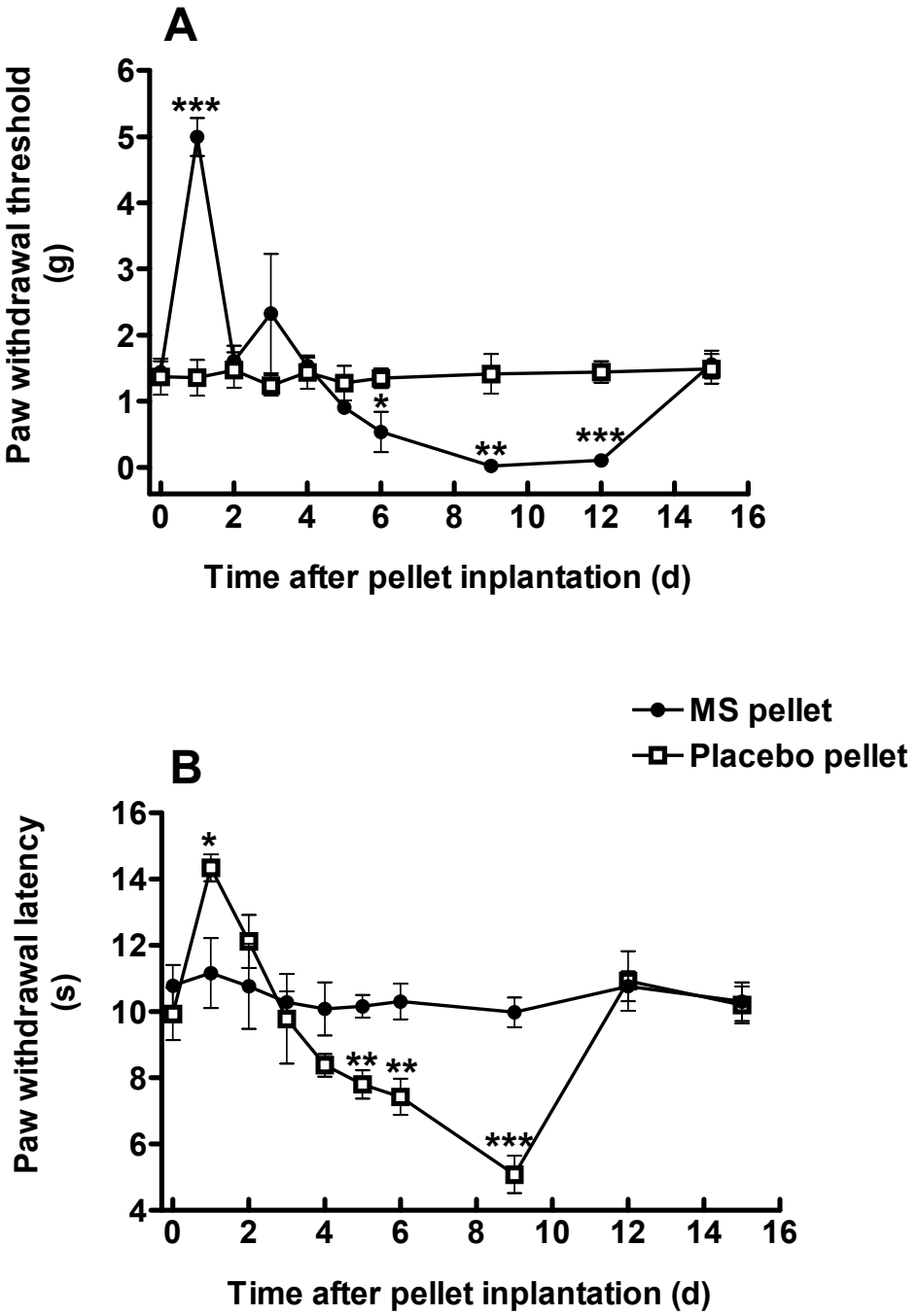


Figure 2. Morphine pellet implantation induced mechanical allodynia (A) and thermal hyperalgesia (B). Mice were implanted subcutaneously with morphine pellet or placebo pellets. The paw withdrawal threshold to von Frey filament probing and withdrawal latency to radiant heat were measured daily. Morphine initially produced antinociception followed by a steep decrease in mechanical threshold (A) and thermal withdrawal latency (B), indicative of the development of OIH. Data are expressed in Mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p < 0.001$, compared with the placebo pellet group; n = 5 for each group.

hyperalgesia was also observed from day 5 to day 9 ($p < 0.001$, compared with placebo group, n = 5) after morphine implantation. Comparing the two OIH models, repeated intermittent morphine administration led to longer lasting and more robust mechanical allodynia and thermal hyperalgesia in ICR mice. Therefore, this model was used for the CaMKII intervention studies.

3.2 CaMKII inhibition by KN93 reversed morphine-induced hyperalgesia

In order to investigate the possible role of CaMKII in OIH, we used KN93, a CaMKII inhibitor, in the initial study. KN92, a kinase inactive analog of KN93, was used as a negative control. Both mechanical allodynia and thermal hyperalgesia were significantly developed on day 5. At that point, mice were treated with KN93 (15 - 45 nmol, i.t.) or KN92 (45 nmol, i.t.) and mechanical and thermal sensitivities were monitored for 24 h. Opioid-induced hyperalgesia was found to be attenuated by KN93 in a dose- and time-dependent manner. At the highest dose, KN93 (45 nmol) completely reversed allodynia and hyperalgesia. Its anti-allodynic/anti-hyperalgesic effect appeared 30 min after KN93 administration and peaked at 2 h (Fig. 3). The antiallodynic/anti-hyperalgesic action of KN93 diminished at 8 h post administration. At lower doses, KN93 (30 nmol, i.t.) partially suppressed allodynia and hyperalgesia, while KN93 at 15 nmol did not affect either thermal or mechanical sensitivity at any time point tested. At the peak-effect time (2 h), ED50 values were estimated to be 26.1 ± 3.4 nmol (anti-allodynic) and 17.8 ± 3.0 nmol (anti-hyperalgesic). In contrast, KN93

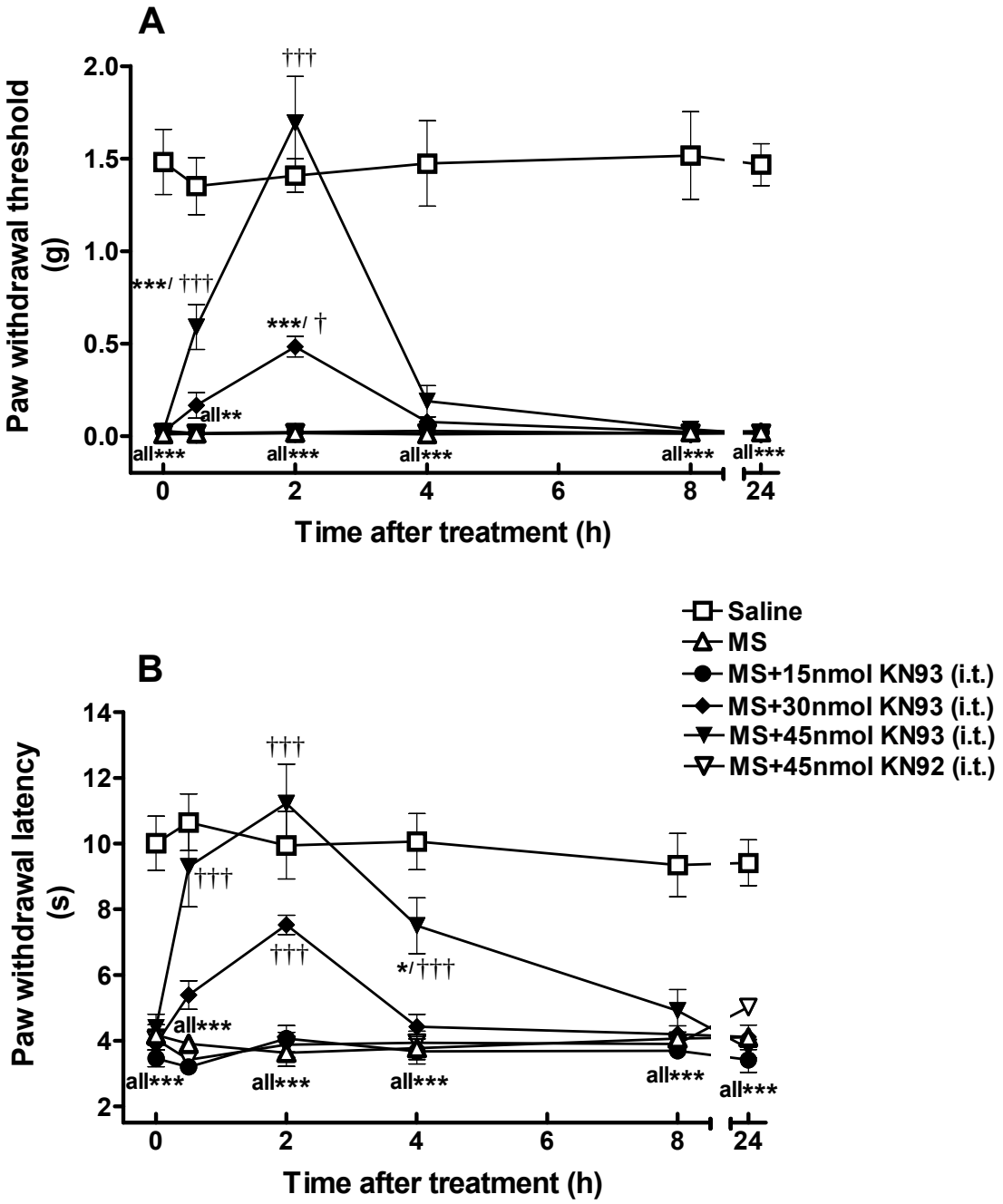


Figure 3. Reversal of morphine-induced mechanical allodynia (A) and thermal hyperalgesia (B) by KN93. OIH was induced by intermittent morphine injections. On day 5, mice received KN93 (15-45 nmol, *i.t.*), KN92 (45nmol, *i.t.*), or saline (*i.t.*) at time 0. Mechanical allodynia and thermal hyperalgesia were tested at the different time points as indicated. KN93, but not KN92, reversed the established morphine-induced mechanical allodynia and thermal hyperalgesia in a dose- and time-dependent manner. Data are expressed in Mean ± SEM. * p<0.05, *** p < 0.001, compared with the saline treated group; † p<0.05, ††† p < 0.001, compared with the morphine treated group; n = 8 for each group.

(45 nmol, i.t.) did not alter the pain threshold at any time point tested, which strongly supported a CaMKII-mediated effort exhibited by KN93. To rule out a potential negative effect of KN93 on locomotor coordination that can be a confounding factor in data interpretation, we further tested the effect of KN93 on locomotor activity in a rotarod test. Administration of KN93 (45 nmol, i.t.) or KN92 (45 nmol, i.t.) to naive mice did not produce significant changes to the locomotor coordination in the rotarod test ($p > 0.05$, compared with saline-treated group, $n = 5$) (Fig. 4). These data are in agreement with our previous observation that KN93 (45 nmol, i.t.) did not change mechanical and thermal sensitivities in naive mice (Luo et al., 2008).

To correlate behavioral effects with biochemical changes, CaMKIIα activity was determined by analyzing the degree of autophosphorylation (pCaMKIIα) (Fang et al., 2002; Xu and Huang, 2004). Compared with the saline control, repeated morphine administration significantly increased spinal pCaMKIIα (Fig. 5A; $p < 0.05$, $n = 4$), without significantly altering its expression (Fig. 5B; $p > 0.05$, $n = 4$). KN93, at the highest dose used (45 nmol, 1 h), significantly reversed the morphine-induced activation of CaMKIIα ($p < 0.05$, compared with morphine group, $n = 4$). In contrast, KN92 (45 nmol) did not change morphine-induced pCaMKIIα. Activation of spinal CaMKII α was also examined using immunohistochemical approach. After chronic morphine exposure, increased pCaMKIIα immunoreactivity was found mostly in the superficial laminae of the spinal dorsal horn compared with the saline-treated mice (Fig. 6). Some pCaMKIIα immunoreactivity was also found in the neuropils which has

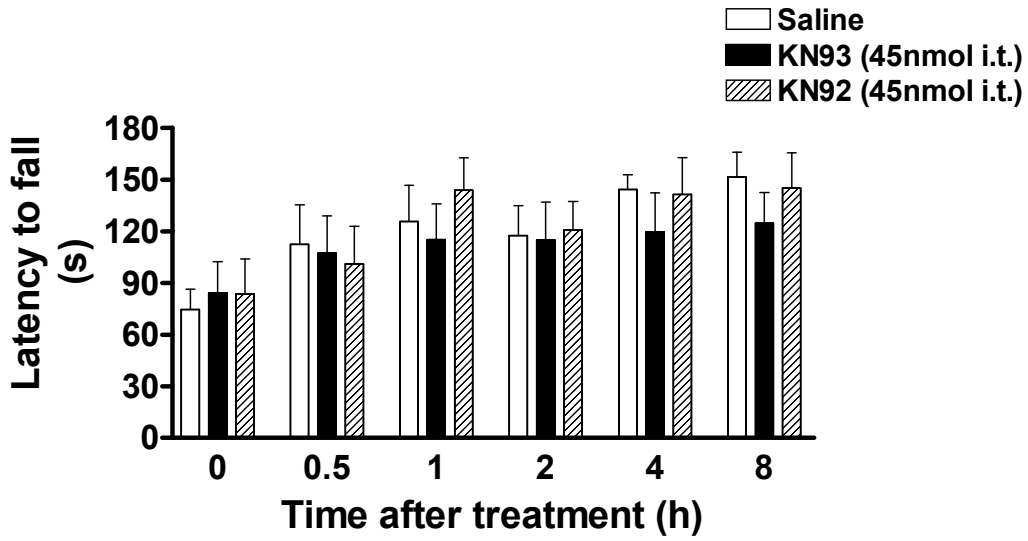


Figure 4. Effect of KN93 on locomotor activity. Mice were trained to remain on a fixed speed (4 rpm) rotarod for 60s. On the following day, each mouse was retrained to ensure that it could stay on the rotarod for at least 60s. Baseline was tested by placing the mice on an accelerating rotarod (4-40 rpm over 300 s). The latency to fall from the rotarod was recorded. Mice were then administered with either KN93 (45 nmol), KN92 (45 nmol) or saline intrathecally and retested 0.5, 1, 2, 4 and 8 h later. At the highest dose used, neither KN93 nor KN92 significantly impaired locomotor activity. Data are expressed in Mean ± SEM. $p > 0.05$, compared with the saline treated group; n=5 for each group.

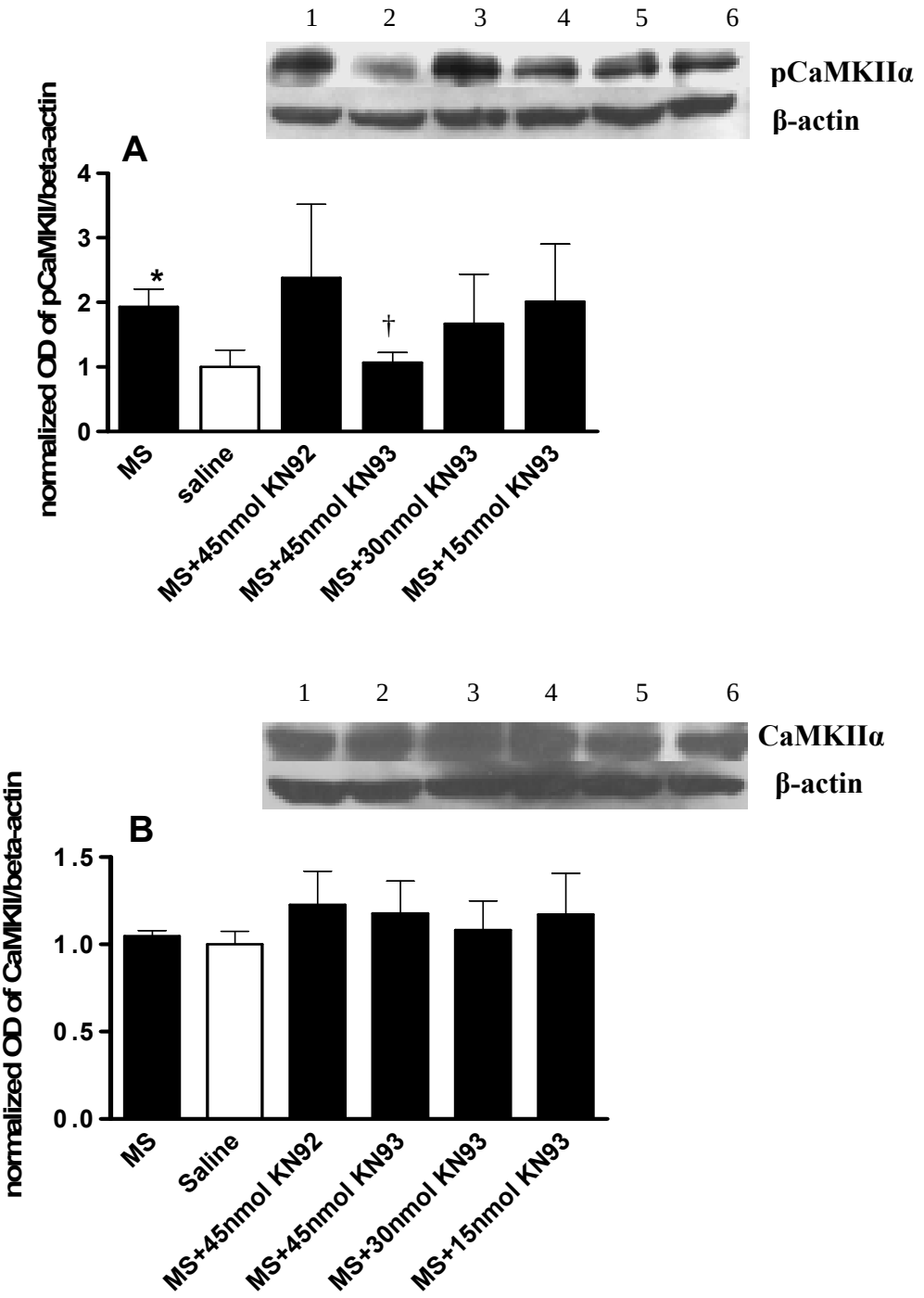
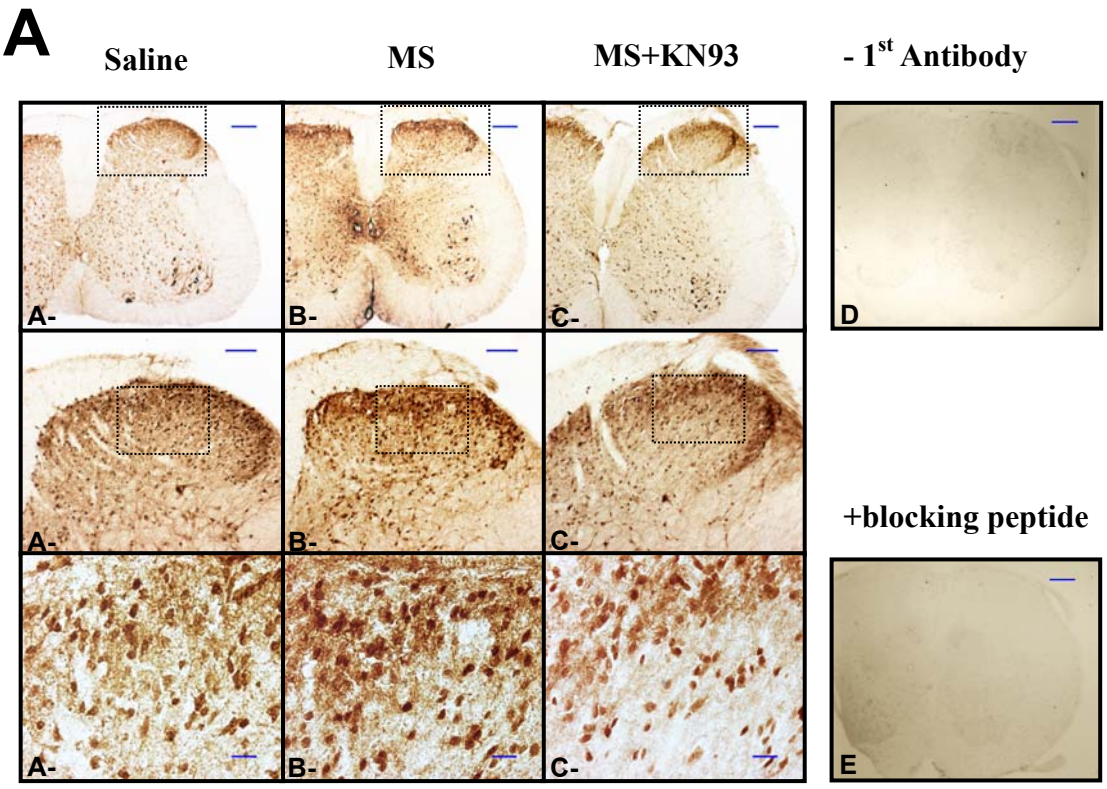


Figure 5. Suppression of morphine-induced CaMKIIα activation by KN93. Morphine or saline treated mice were administered (i.t.) with KN93 (15-45nmol), KN93 (45nmol), or saline on day 5. One hour later, mice were sacrificed and the lumbar spinal cords were taken for the analysis of CaMKIIα activation using the immunoblotting method, by determining the degree of CaMKIIα autophosphorylation (pCaMKIIα). Morphine enhanced pCaMKIIα expression, without altering CaMKIIα expression. KN93, but not its inactive analog, KN92, reversed morphine enhanced CaMKIIα activation. Data are expressed in Mean ± SEM. * $p < 0.05$, compared with the saline treated group; † $p < 0.05$, compared with the morphine treated group; n = 4 for each group

been reported by others as well (Liang et al., 2004; Fujiyoshi et al., 2007; Song et al., 2009). However, the enhanced pCaMKIIα immunostaining was significantly suppressed by KN93 (45 nmol, i.t.) (Fig. 6B). These data suggested that chronic morphine-induced mechanical allodynia and thermal hyperalgesia, and their reversal by KN93, are biochemically correlated with morphine-induced CaMKIIα activation and subsequent inactivation in the presence of KN93, but not KN92.

3.3 Small interfering RNA (siRNA) -mediated knockdown of CaMKIIα and reversal of morphine- induced hyperalgesia

To further investigate the specific isoform of CaMKII involved in OIH, siRNA targeting CaMKIIα (Zayzafoon et al., 2005) was applied to knock down the expression of CaMKIIα in the spinal cord. Mice received repeated morphine administration in phase 1 (Fig. 7) and, as expected, developed mechanical allodynia and thermal hyperalgesia 18 h after the final morphine injection. In phase 2, mice received intrathecal administration of CaMKIIα siRNA or scrambled siRNA (2 μg/injection, twice per day for 3 consecutive days) and the sensitivities to mechanical and thermal stimuli were measured daily. Treatment with CaMKIIα siRNA gradually attenuated morphine-induced mechanical allodynia (Fig. 7A) and thermal hyperalgesia (Fig. 7B). After 3 d of treatment, OIH was completely reversed (Fig. 7, $p < 0.001$, compared with


A
Saline
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MS+KN93
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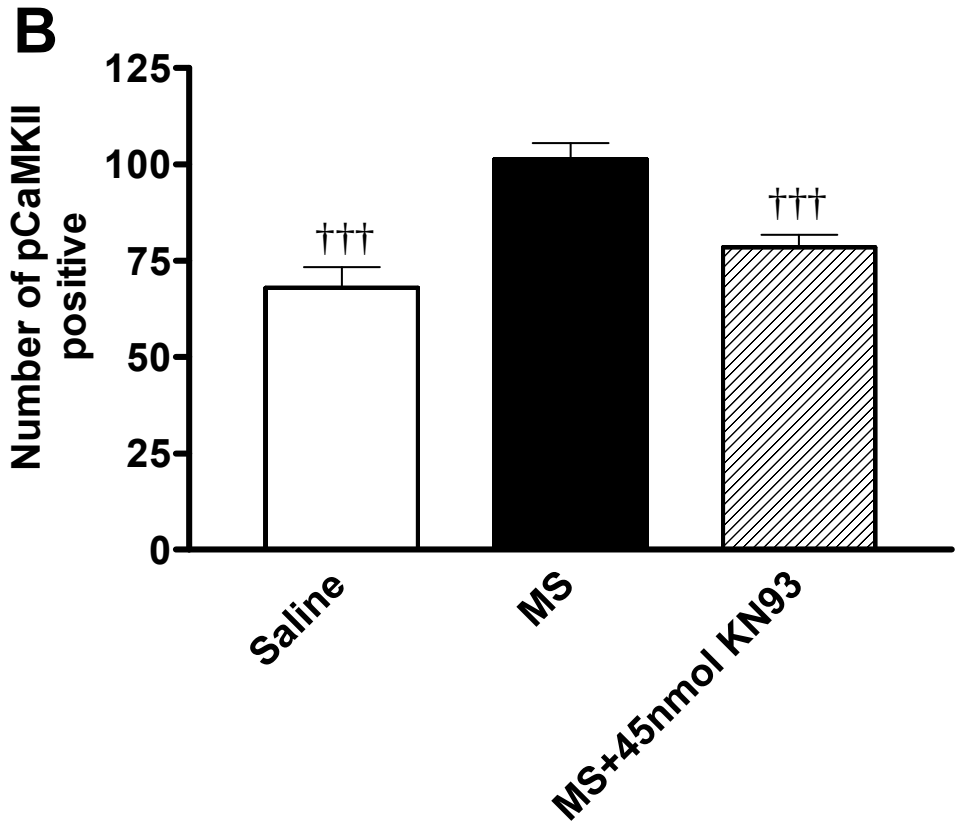

B
Number of pCaMKII positive
125
100
75
50
25
0
†††
†††
Saline
MS
MS+45nmol KN93

Figure 6. Immunohistochemical staining of pCaMKIIα expression after the treatment with KN93. Morphine or saline treated mice were administered *(i.t.)* with KN93 (15-45nmol), KN93 (45nmol), or saline on day 5. Mice were sacrificed 1h after the treatment with saline or KN93 (45nmol, *i.t.*). The lumber spinal section was dissected out and immunostained with pCaMKIIα antibody. Quantitative analysis of pCaMKIIα immunoreactivity was performed by counting the number of positively stained cells using the MetaMorph Imaging Software. No pCaMKIIα immunoreactivity was detected if the 1st antibody was omitted (D) or if the 1st antibody was incubated in the presence of pCaMKIIαT286 blocking peptide (Santa Cruz). Data are expressed in Mean ± SEM. †††, $p<0.001$, compared with the morphine treated group; n=3 for each group. Scale bars are 200μm (A-1; B-1; C-1; D; E), 100μm (A-2; B-2; C-2), or 20μm (A-3; B-3; C-3).

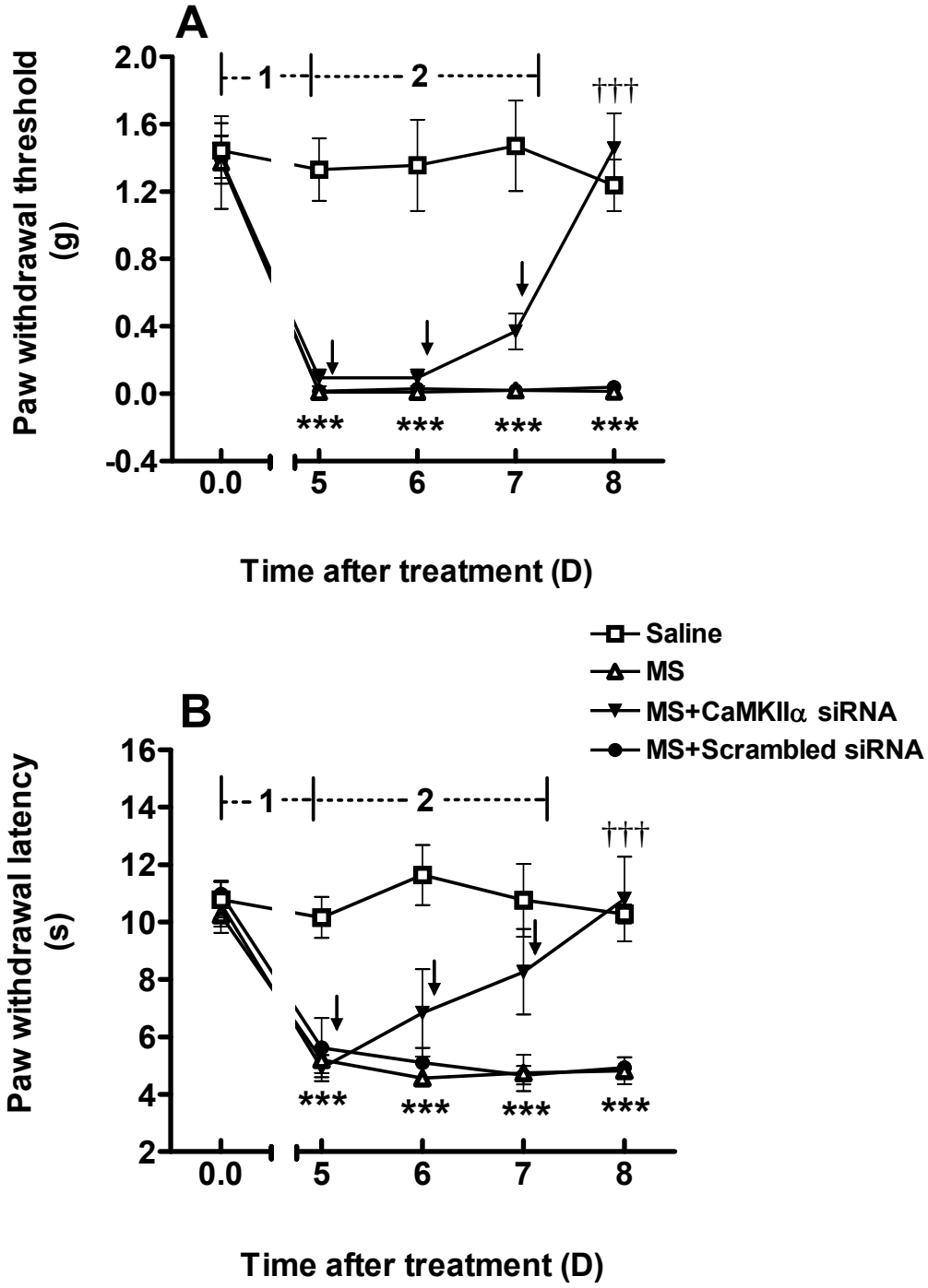
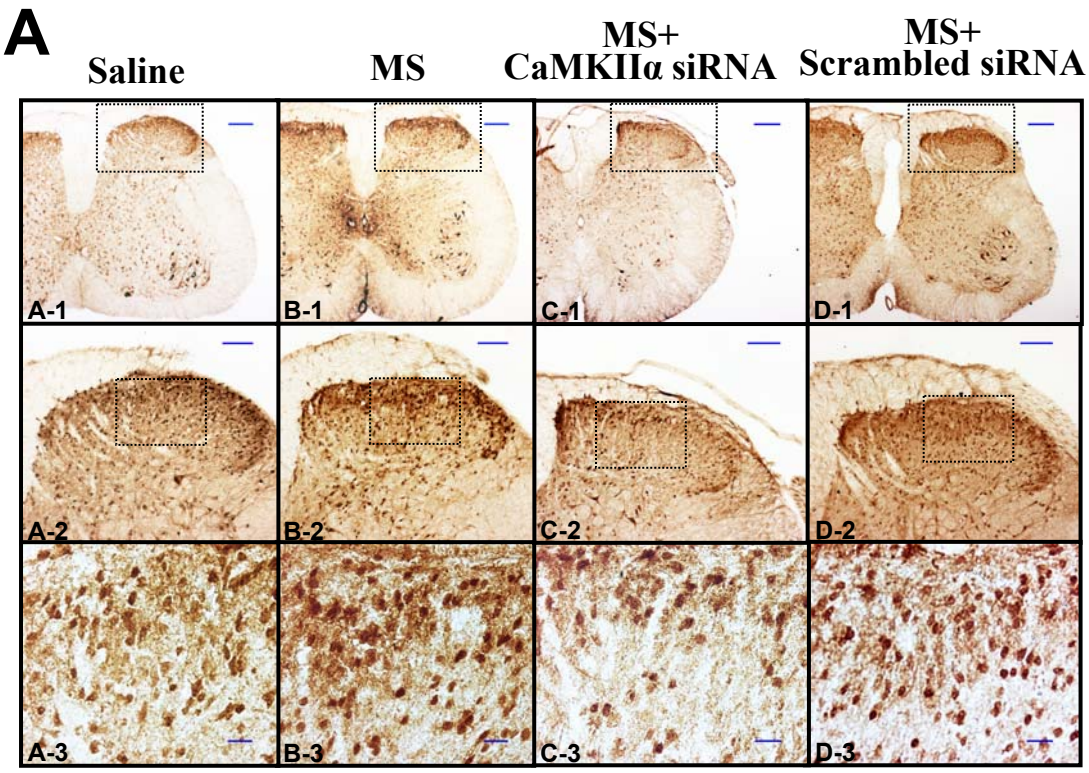


Figure 7. Reversal of OIH by siRNA-mediated CaMKIIα knockdown. In phase 1, mice received repeated saline or morphine administration. Mechanical and thermal sensitivities were measured on day 5. In phase 2, after OIH had fully developed, mice were treated with CaMKIIα or scrambled siRNA (2μg, twice/day for 3 days.). Mechanical and thermal sensitivities were tested daily. Established OIH was reversed by CaMKIIα siRNA. Data are expressed in Mean ± SEM. *** p<0.001, compared with the saline treated group; ††† p < 0.001, compared with the morphine treated group; n = 5 for each group. Arrows indicated saline, CaMKIIα or scrambled siRNA administration.

morphine-treated group, n = 5). Correlating with the behavioral effect, pCaMKIIα immunostaining in the spinal dorsal horn was significantly suppressed (Fig. 8). In contrast, scrambled siRNA did not alter OIH (Fig. 7) or morphine-induced pCaMKIIα immunoreactivity (Fig. 8).

3.4 Absence of morphine-induced hyperalgesia in CaMKIIα^{T286A} point mutation mice

To investigate whether functional CaMKIIα is also required for the development of OIH, we used CaMKIIα^{T286A} mice in the study. These mice were engineered to carry a T286A point mutation, lacking the ability to undergo CaMKIIα autophosphorylation and further activation (Giese et al., 1998). We investigated whether inactivation of CaMKIIα by T286A point mutation affected morphine-induced hyperalgesia. Separate group of 6 male CaMKIIα^{T286A} mutant and littermate wild-type mice received repeated injections of morphine every 12 h for 4 d using the same treatment schedule as described above. Baseline pain thresholds were not significantly different between wild-type and mutant mice (Fig. 9). The wild-type mice exhibited mechanical allodynia and thermal hyperalgesia on day 5, indicative of the presence of OIH ($p < 0.01$, compared with baseline, n = 6). However, mechanical allodynia and thermal hyperalgesia were not detected in morphine-treated CaMKIIα^{T286A} mutant mice. These data indicated that CaMKIIα is required for the initiation of OIH (Fig. 9).


A
Saline
MS
MS+
CaMKIIα siRNA
MS+
Scrambled siRNA
A-1
B-1
C-1
D-1
A-2
B-2
C-2
D-2
A-3
B-3
C-3
D-3

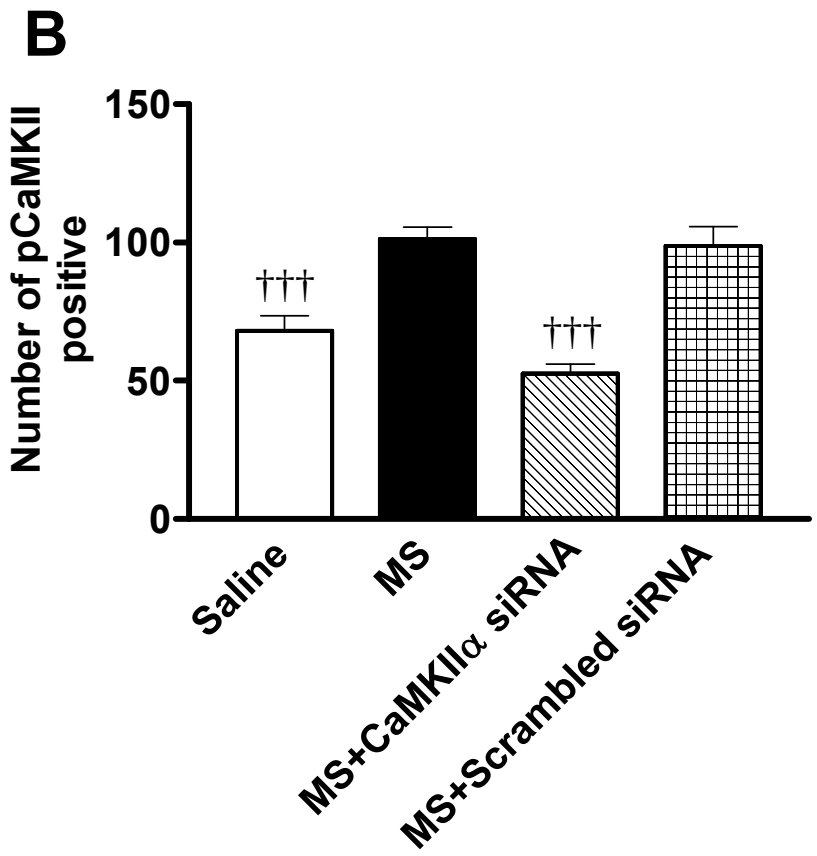

B
Number of pCaMKII positive
150
100
50
0
†††
†††
Saline
MS
MS+CaMKIIα siRNA
MS+Scrambled siRNA

Figure 8. Suppression of morphine induced CaMKIIα activation by CaMKIIα siRNA. Naïve mice (A) or mice with OIH were treated with saline (B), CaMKIIα siRNA (2μg, twice/day for 3 days) (C), or scrambled siRNA (2μg, twice/day for 3 days). One day after the last injection of siRNA, the spinal lumber region was immunostained with pCaMKIIα antibody. Scale bars are 200μm (A-1; B-1; C-1; D-1), 100μm (A-2; B-2; C-2; D-2), or 20μm (A-3; B-3; C-3; D-3). Quantitative analysis of pCaMKIIα immunoactivity was performed by counting the number of positively stained cells using MetaMorph Imaging Software. Data are expressed in Mean ± SEM. ††† $p<0.001$, compared with the morphine treated group; n=3 for each group.

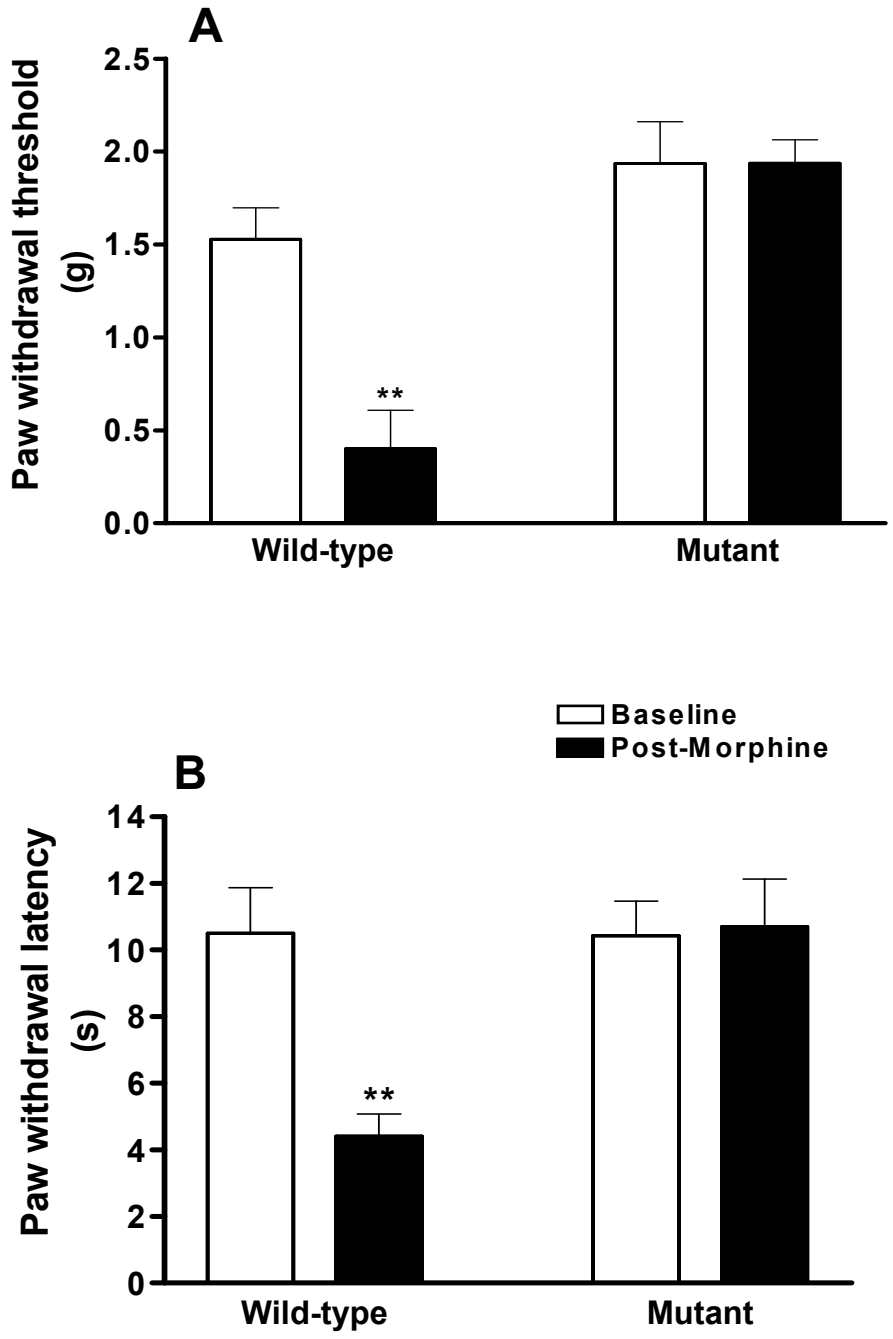


Figure 9. Morphine failed to induce hyperalgesia in CaMKIIα^{T286A} mutant mice. Male CaMKIIα^{T286A} mutant and littermate wild-type mice were subcutaneously administered saline or morphine sulfate (day 1-3: 20 mg/kg/day; day 4: 40 mg/kg, twice daily). Baseline pain thresholds were not significantly different between wild-type and mutant mice. Mechanical and thermal sensitivities were tested on day 5. Morphine treatment successfully induced hyperalgesia in CaMKIIα$^{T286A+/+}$ mice (** $p < 0.01$, compared with the baseline). However, CaMKIIα$^{T286A-/-}$ mice failed to develop OIH ($p > 0.05$, compared with the baseline). Data are expressed in Mean ± SEM; n=6 for each group.

4. Discussion

In this study, we tested the hypothesis that CaMKIIα is a required component for the development and maintenance of OIH. We found that acute CaMKII inhibition by KN93 or siRNA-mediated knockdown of CaMKIIα reversed opioid-induced mechanical allodynia and thermal hyperalgesia. OIH was absent in CaMKIIα^{T286A} mutant mice. Comparing two mouse models of OIH, the intermittent injection method offered longer lasting and more robust OIH compared with the pellet model, and was chosen as the model for CaMKII intervention studies. CaMKII inhibition by KN93 reversed OIH, correlating with decreased CaMKIIα activation by KN93 in the spinal cord. In contrast, the kinase-inactive control compound KN92 did not affect CaMKIIα activity or OIH. In the previous studies, we found that KN92 did not inhibit other isoforms of CaMKII, either (Tang et al., 2006a; Luo et al., 2008; Chen et al., 2009). The methoxybenzenesulfonamide KN93 is the most commonly used CaMKII inhibitor. It does not inhibit protein kinase C, protein kinase A, or even other CaM-dependent enzymes such as MLCK or CaM-dependent phosphodiesterase at very high concentrations (Sumi et al., 1991; Niki et al., 1993). Due to its wide applications, however, several nonspecific actions have been reported for the compound. For example, both KN93 and KN92 blocked voltage-dependent potassium channels in vascular myocytes (Ledoux et al., 1999; Rezazadeh et al., 2006) and L-type calcium channels in SH-SY5Y cells (Gao et al., 2006). That is why we have applied the negative control, KN92, at a high dose in all studies involving KN93. The isoquinolinesulfonamide derivative, KN62, another CaMKII inhibitor, has been found to

act as an antagonist of the P2X7 receptor (Chessell et al., 1998; Baraldi et al., 2003), although such a CaMKII-independent effect has not been reported for KN93 or KN92.

To ascertain a CaMKII-mediated mechanism and to investigate the specific isoform involved, we further targeted CaMKIIα by genetic approaches. Knockdown of CaMKIIα by siRNA and resultant suppression of OIH suggested that CaMKIIα was the relevant isoform. These studies were controlled by scrambled siRNA to exclude off-target interference. Some siRNA molecules have been reported to cause nonspecific interferon response (Bridge et al., 2003; Sledz et al., 2003; Hornung et al., 2005), leading to nonspecific inhibition of protein synthesis and nonspecific degradation of endogenous mRNA. In a separate study, we tested the effect of CaMKIIα siRNA on the expression of five key genes: OAS1, OAS2, MX1, ISGF3γ and IFITM1 that are involved in the interferon response (Veals et al., 1992; Deblandre et al., 1995; Hovnanian et al., 1998; Kochs et al., 2002) and found that the sequence did not produce an interferon response. Moreover, lack of morphine-induced hyperalgesia in CaMKIIα$^{T286A-/-}$ mice further indicated that CaMKIIα is required for the initiation of OIH. These data, for the first time, implicated a critical role of CaMKIIα in OIH.

In addition to CaMKIIα , several other mechanisms underlying OIH have been suggested, such as the β2-adrenergic receptor (Liang et al., 2006), spinal cyclooxygenase (COX) (Dunbar et al., 2000), as well as local cytokine production (Liang et al., 2008). It has been suggested that both peripheral and central sensitization are important for the development of OIH (Chu et al., 2008). Blockade of the *N*-methyl-D-aspartate (NMDA) receptor by MK801 or ketamine has been shown to

reverse (Li et al., 2001b) or prevent (Rivat et al., 2002) OIH. A vast amount of information is available for the interaction of CaMKIIα and the NMDA receptor, although these data are mostly from learning and memory studies. Both CaMKIIα and the NMDA receptor are required for the generation of long-term potentiation (LTP) in hippocampal neurons (Mayford et al., 1996). Inhibition of CaMKII or deletion of the CaMKII gene impairs spatial learning (Silva et al., 1992; Wolfman et al., 1994; Silva and Josselyn, 2002). It has been demonstrated that Ca^{2+} influx via the activation of NMDA receptors results in CaMKIIα activation and autophosphorylation of CaMKIIα at position Thr286 (Fukunaga et al., 1992; Strack et al., 2000). Cytosolic free Ca^{2+} was increased after treatment with opioids (Smart et al., 1997; Spencer et al., 1997; Quillan et al., 2002). We and others have demonstrated that CaMKIIα activity is increased after chronic treatment with opioids (Tang et al., 2006a). On the other hand, CaMKIIα can phosphorylate and activate the NMDA receptor, leading to the influx of Ca^{2+} through the channels (Kitamura et al., 1993). Therefore, CaMKII and the NMDA receptor may interact in a feedforward manner in OIH. Such a mechanism may explain the persistent nature of OIH, especially if one considers that desensitization can occur to a protein kinase after prolonged activation.

The μ-opioid receptor is another potential substrate of CaMKIIα. The receptor is expressed in the primary afferent neurons and is colocalized with CaMKII in pain-processing regions such as dorsal root ganglion sensory neurons and superficial layers of the spinal dorsal horn (Bruggemann et al., 2000; Carlton, 2002). The interaction between CaMKIIα and the μ-opioid receptor was further supported by

cellular studies. The μ-opioid receptor contains consensus CaMKIIα phosphorylation sites. Desensitization to the μ-opioid receptor was enhanced when a constitutively active CaMKIIα was expressed in *Xenopus* oocytes (Mestek et al., 1995; Koch et al., 1997), which was abolished if the receptor was mutated to delete the putative CaMKIIα phosphorylation sites (Koch et al., 1997). In addition to the NMDA receptor and the μ-opioid receptor, there are likely many other potential substrates for CaMKIIα and it will be imperative to identify these targets in future studies to better understand and ultimately treat OIH. Such searches may benefit from increasingly available proteomics tools.

Both CaMKII and PKC are important kinases mediating Ca^{2+} signaling. Ca^{2+} influx through ion channels such as the NMDA receptor results in the activation of CaMKII and PKC. Enhanced activation of PKC has been reported in OIH (Celerier et al., 2004). Opioid-induced hyperalgesia is absent in mice lacking the gene coding for PKCγ (Celerier et al., 2004). It will be very interesting to study the possible crosstalk between CaMKII and PKC in OIH. The NR2A subunit of the NMDA receptor serves as the CaMKIIα docking site that binds to autophosphorylated CaMKIIα, a process that is known to be regulated by PKC (Gardoni et al., 2001; Leonard et al., 2002). Recently, we have identified the neuronal PKC substrate, neurogranin, as a mechanism that synchronizes the action of CaMKII and PKC in opioid tolerance and dependence (Shukla et al., 2006; Tang et al., 2006b; Tang et al., 2007b).

In summary, our data strongly implicate CaMKIIα as a cellular mechanism leading to and maintaining opioid-induced hyperalgesia. Accumulating evidence

highlights an important role for CaMKII in morphine tolerance and dependence (Tang et al., 2006a), CFA-induced inflammatory pain (Luo et al., 2008), and spinal nerve injury-induced neuropathic pain (Chen et al., 2009). These findings add to the growing literature that OIH, opioid tolerance, opioid dependence, and neuropathic pain may share common mechanisms at a certain level, although they are distinct phenomena (Mayer et al., 1999; Horvath and DeLeo, 2009). Targeting CaMKII may be useful for the prevention and treatment of OIH.

V. SUMMARY AND FUTURE STUDIES

Although pain, especially chronic pain, has been a pervasive health problem that effects the patient, their significant others, and society in many ways, the study of underlying mechanisms remain unclear. To study the mechanism of inflammatory and neuropathic pain, several pain models in rodents including CFA-induced inflammatory pain, SNL-induced neuropathic pain, and opioid-induced hyperalgesia were used here. First, we evaluated the role of CaMKII inhibition by inhibitor KN93 in all the models and found both pain behavior and evaluated CaMKII activity during pain processing were significantly reversed by KN93. Furthermore, we previously identified trifluoperazine, a clinically used antipsychotic drug, to be a potent CaMKII inhibitor. In this study, inhibition of CaMKII activity by trifluoperazine was also confirmed.

To elucidate which isoform of CaMKII is involved, we especially knock-down CaMKIIα using siRNA in mice with developed OIH. Both mechanical allodynia and thermal hyperalgesia induced by repeated morphine injection was alleviated when CaMKIIα function was impaired. Furthermore, experiments using CaMKIIαT286A mice suggested that CaMKIIα is also required in pain initiation step. In conclusion, the findings described above provide new insight into the signaling mechanisms of CaMKIIα in inflammatory and neuropathic pain. The pharmacological study of CaMKIIc inhibition may open the door for novel analgesics effective drugs for pain management.

One of the challenges for studying chronic pain is that it may involve a mix of both

inflammatory and neuropathic components. For instance, inflammation induced release of cytokines and growth factors may have direct neural activity or may cause damage to the neurons and produce neuropathic pain. On the other hand, neural injury may cause an inflammatory reaction and contributes to the inflammatory pain. Here we demonstrated that CaMKIIα may serve as a common mechanism shared by both pain conditions. So pharmacologically inhibition of CaMKIIα may solve the complicated situation using a simple way. Our future study may focus on studying the regulation of CaMKIIα and its entire signaling pathway involved in pain condition.

To make the case more complicate, people believe that pain is not a simple a physiological disease, a more complete understanding of pain must take into account with psychological effects and even social factors. However, the study of pain in such a psychological level can not be achieved on animal models. So clinical reports of pain management in patients will highly enrich our understanding. Also, study of FDA-approved drugs, such as trifluoperazine used in our study, will provide a new direction for clinical pain management.

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UNIVERSITY OF ILLINOIS
AT CHICAGO

Office of Animal Care and Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

July 22, 2008

Zaijie Jim Wang
Biopharmaceutical Sciences
M/C 865

Dear Dr. Wang:

The protocol or modifications indicated below has been **terminated** in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago on **07/21/2008** for the following checked reason:

ACC No: **05-136**

Title of Application: **Drug Modulation of Pain and Opiod Analgesia**

[ ] PI no longer at UIC
[ ] Confirmation of the PI's request to terminate the above referenced protocol and/or modification
[X] The work covered by the protocol for animal use referenced above is now covered by the following animal care protocol **08-119**
[ ] Protocol/modification was approved for only one year.
[ ] Failure to respond to the annual continuation notices
[ ] Failure to respond to the resubmission notices
[ ] Failure to respond to the veterinarian pre-review concerns within 60 days of protocol submission
[ ] Failure to respond to the Animal Care Committee concerns within 60 days of protocol review

If you have any further questions please do not hesitate to call the Animal Care Office at (312) 996-1972. Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Mariann R Piano

Mariann R. Piano, PhD
Chair, Animal Care Committee

MRP/*KG*
cc: BRL, ACC File

VITA

NAME： Yan Chen

EDUCATION:

09/2006-present, Graduate student in Ph.D. program, Department of biopharmaceutical sciences, **University of Illinois at Chicago**.

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Graduate student in Ph.D. program, Department of biological science, **Wayne State University**.

09/2002-06/2004, M.S., Majoring in Pharmaceutical science, Department of Biology, **Nanjing University**

09/1998-07/2002, B.S., Majoring in Biology, Department for Intensive Instruction for Fundamental Science, **Nanjing University**

HONORS AND SCHOLARSHIPS:

1. 2010 Dean's Scholarship in University of Illinois at Chicago
2. 2010 W.E. van Doren Scholarship in college of pharmacy
3. 2010 American Pain Society Travel Award 2010
4. 2009 Myron Goldsmith Scholarship in college of pharmacy, UIC
5. 2009 UIC University fellowship
6. 2009 American pain society annual meeting Travel Award
7. 2009 UIC Graduate College Travel Award
8. 2009 International Narcotics Research Conference Travel Award
9. 2008 Membership in Golden Key International Honour Society
10. 2008 W.E. van Doren in college of pharmacy, UIC
11. 2008 Membership in Rho Chi pharmacy Honour Society
12. 2008 International Narcotics Research Conference Travel Award
13. 2008 UIC Graduate College Travel Award
14. 2007 UIC Women in Science and Engineering Program Motorola Foundation Travel Grants.
15. 2007 UIC Graduate Student Council Travel Award
16. 2006 UIC University fellowship
17. 2003 Privilege to acquire M.S. degree ahead of one year
18. 2002 Privilege to enter the Graduate program at Nanjing University, waived of the admission test
19. 2001 People’s Scholarship second Prize (top 10%)
20. 1998 People’s Scholarship second Prize (top 10%)
21. 1998 Privilege to enter Nanjing University, waived of the National Entrance Examination

Research and Teaching Assistant, University of Illinois Chicago. 2005-2010

PUBLICATIONS:

1 **Y Chen,** Cheng Yang, and Zaijie Jim Wang， A behavioral screen identifies PKC isoforms in SNL-induced pain. *Neuroscience.* In progress.

2 **Y Chen,** Cheng Yang, and Zaijie Jim Wang, Proteinase-activated receptor 2 sensitizes TRPV1, TRPV4 and TRPA1 in paclitaxel-induced neuropathic pain. *Neuroscience.* Accepted.

3 C Yang, **Y Chen,** L Tang and ZJ Wang, Haloperidol disrupts opioid antinociceptive tolerance and physical dependence. *J Pharmacol Exp Ther,* 2011 Jul;338(1):164-72.

4 **Y Chen**, C Yang, and ZJ Wang. Ca2+/Calmodulin-Dependent Protein Kinase Iiα Is Required for the Initiation and Maintenance of Opioid-Induced Hyperalgesia. *J Neuroscience*, 2010, 30(1):38-46.

5 **Y Chen,** F Luo, C Yang, CM Kirkmire, and ZJ Wang. Acute Inhibition of Ca2+/Calmodulin-Dependent Protein Kinase II Reverses Experimental Neuropathic Pain in Mice. *J Pharmacol Exp Ther*, 2009, 330: 650-9.

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8 **Y Chen**, HD Wang, X Xia, HF Kung, Y Pan and LD Kong, Behavioral and Biochemical Studies of Total Furocoumarins from Seeds of Psoralea Corylifolia in The Chronic Mild Stress Model of Depression in Mice. *Phytomedicine,* 2007, 14(7-8):523-9.

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11 Yu ZF, Kong LD and **Chen Y**. Antidepressant Activity of Aqueous Extracts of Curcuma Longa in Mice. J Ethnopharmacol. 2002 Nov;83(1-2):161-5.

12 ZF Yu, C Yang, X Qiu, **Y Chen**, LD Kong and Mingshi Wang, Effects of Daphnin on Serum Uric Acid Level in in vivo Model of Hyperuricemic Mice. *Journal of China Pharmaceutical University*, 2002, 33(2):142~145.

MEETINGS ATTENDED:

1. American Pain Society Annual Meeting, Baltimore, MD (May. 2010)
2. International Narcotics Research Conference. Portland, OR (Jul. 2009).

3. 41th Pharmaceutics Graduate Student Research Meeting, West Lafayette, IN (Jun. 2009).
4. American Pain Society Annual Meeting, San Diego, CA (May. 2009)
5. 2008 Neuroscience Day Meeting, Chicago, IL (Dec. 2008).
6. International Narcotics Research Conference. Charleston, SC (Jul. 2008).
7. 40th Pharmaceutics Graduate Student Research Meeting, Ann arbor, MI (Jun. 2008).
8. Chicago Chapter Society for Neuroscience Meeting, Chicago, IL (Mar. 2008).
9. American Association of Pharmaceutical Scientists Annual Meeting, San Diego, CA (Nov. 2007).
10. 39th Pharmaceutics Graduate Student Research Meeting, Kansas City, MO (Jun. 2007).

POSTERS:

1. **Y.Chen**, C,Yang, Z.J.Wang, CaMKIIα as a common mechanism mediating opioid tolerance and hyperalgesia. International Narcotics Research Conference, . Portland, OR (Jul. 2009).
2. **Y.Chen**, C,Yang, F,Luo, Z.J.Wang, CaMKIIα, a common mediator of neuropathic pain and opioid induced hyperalgesia. American Pain Society Annual Meeting, San Diego, CA (May. 2009)
3. **Y.Chen**, C,Yang, F,Luo, L.Tang, Z.J.Wang, CaMKII as a common mechanism mediating opioid tolerance and hyperalgesia. Neuroscience Day Meeting, Chicago, IL, (Dec. 2008).
4. C. Yang, **Y. Chen**, L. Tang, Z.J. Wang, Antipsychotic Drug Haloperidol Disrupts Opioid Antinociceptive Tolerance and Dependence via CaMKII. Neuroscience Day Meeting, Chicago, IL, (Dec. 2008).
5. **Y.Chen,** C,Yang, F,Luo, L.Tang, Z.J.Wang, CaMKII as a common mechanism mediating opioid tolerance and hyperalgesia. International Narcotics Research Conference, Charleston, SC (Jul. 2008).
6. C. Yang, **Y. Chen**, L. Tang, Z.J. Wang, Antipsychotic Drug Haloperidol Disrupts Opioid Antinociceptive Tolerance and Dependence via CaMKII. International Narcotics Research Conference, Charleston, SC (Jul. 2008).
7. **Y. Chen**, C.Yang, F.Luo, L.Tang, Z.J.Wang, Reversal of chronic neuropathic pain by a cute inhibition of CaMKII. 40th Pharmaceutics Graduate Student Research Meeting, Ann arbor, MI (Jun. 2008).
8. Fang Lou, Cheng Yang**, Yan Chen**, Zaijie J.Wang. Reversal of chronic inflammatory pain by acute inhibition of Ca2+/Calmodulin-depedent protein kinase II. 40th Pharmaceutics Graduate Student Research Meeting, Ann arbor, MI (Jun. 2008).
9. **Y.Chen,** L.Tang, P.Shukla, Z.J.Wang, Antinociceptive Pharmacology of N-(4-Chlorobenzyl)-N'-(4-hydroxy-3-iodo-5-methoxybenzyl) Thiourea, a High-Affinity Competitive Antagonist of the Transient Receptor Potential Vanilloid

1 Receptor. Chicago Chapter Society for Neuroscience Meeting, Chicago, IL (Mar. 2008).

10. Fang Lou, Cheng Yang, **Yan Chen,** Pradeep Shukla,Lei Tang,Lili X.Wang,Zaijie J.Wang. Reversal of chronic inflammatory pain by acute inhibition of Ca2+/Calmodulin-dependent protein kinase II. Chicago Chapter Society for Neuroscience Meeting, Chicago, IL (Mar. 2008).
11. **Y.Chen**, L.Tang, P.Shukla, Z.J.Wang, Antinociceptive Parmacology of N-(4-Chlorobenzyl)-N'-(4-hydroxy-3-indo-5-methoxybenzyl) Thiourea, a High-Affinity Competitive Antagonist of the Transient Receptor Potential Vanilloid 1 Recetor. American Association of Pharmaceutical Scientists Annual Meeting, San Diego, CA (Nov. 2007).
12. Lu J, Yang C, **Chen Y**, Luo F, Lee B, Onyuksel H, Wang ZJ. In vitro and in vivo studies of dermorphin-conjugated sterically stabilized liposomes: potential application in pain and addiction therapies. AASP San Diego, CA (Nov. 2007).
13. **Y.Chen**, L.Tang, P.Shukla, Z.J.Wang, Antinociceptive Parmacology of N-(4-Chlorobenzyl)-N'-(4-hydroxy-3-indo-5-methoxybenzyl) Thiourea, a High-Affinity Competitive Antagonist of the Transient Receptor Potential Vanilloid 1 Recetor. 39th Pharmaceutics Graduate Student Research Meeting, Kansas City, MO (Jun. 2007).