Journal of Inflammation Research

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ORIGINAL RESEARCH

The κ-Opioid Receptor Agonist U50488H Ameliorates Neuropathic Pain Through the Ca^{2+}/CaMKII/CREB Pathway in Rats

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Objective: To observe the ameliorative effect of kappa opioid receptor (KOR) agonist on rats with neuropathic pain (NP) and investigate the mechanism of action of the calcium ion (Ca^{2+})/calcium/calmodulin-dependent protein kinase II (CaMKII)/cyclic AMP response element-binding protein (CREB) pathway.

Methods: A total of 40 Sprague Dawley rats were randomly divided into four groups: sham-operation group (Sham group), NP model group (NP group), NP + KOR agonist U50488H group (NU group) and NP + specific CaMKII antagonist (KN93) + U50488H group (NKU group). The thermal withdrawal latency (TWL) and mechanical withdrawal threshold (MWT) of each group of rats were determined. ELISA was applied to examine the changes in inflammatory factors and oxidative stress factors, and the apoptotic rate in dorsal root ganglia was observed using TUNEL staining. Ca^{2+} concentration, content of oxidative stress index ROS and the release of calcitonin gene-related peptide (CGRP) and N-methyl-D-aspartate receptor (NMDAR) in the dorsal root ganglia were measured by the immunofluorescence assay. Finally, Western blotting was performed to detect expression changes in the Ca^{2+}/CaMKII/CREB pathway.

Results: The KOR agonist U50488H could improve the values of TWL and MWT of NP the rats, inhibit inflammatory responses and relieve oxidative stress injury. Its mechanisms of action were associated with U50488H repression of Ca^{2+} influx, reduction of CGRP and NMDAR releases in the dorsal root ganglia and decreases in CaMKII and CREB phosphorylations in NP rats.

Conclusion: The KOR agonist ameliorates NP through suppressing the activity of the Ca^{2+}/CaMKII/CREB pathway.

Keywords: KORs, neuropathic pain, Ca^{2+}, CaMKII, CREB

Introduction

Neuropathic pain (NP) is directly caused by injury or disease of the somatosensory nervous system and it mainly manifests as allodynia, spontaneous pain and hyperalgesia.[1] It is a severe and refractory disease that results in tremendous economic, social and psychological burdens to patients. In recent years, studies have revealed that large quantities of immune cells migrate to the site of neural injury, where they synthesize and release inflammatory factors that can interact with neurotransmitters [P-selectin, calcitonin gene-related peptide (CGRP) and prostaglandin] and their receptors [N-methyl-D-aspartate receptor (NMDAR), AMPAR and opioid receptors], released by neurons and glial cells to activate multiple

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Received: 9 August 2021
Accepted: 2 November 2021
Published: 23 May 2022

intracellular signal transduction pathways, that regulate immune responses and pain excitability conduction pathways.[2]

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a multi-functional serine/threonine protein kinase, that is primarily distributed in spinal dorsal horns and dorsal root ganglia, where it plays an important role in the transmission of noxious stimuli in the spinal cord postsynaptic interneurons.[3] During neural injury, NMDAR is overactivated and the permeability of cell membrane to calcium ion (Ca^{2+}) is increased, which lead to a large amount of Ca^{2+} influx that promotes cellular calcium overload. Subsequently, Ca^{2+} binds to CaMKII and triggers the phosphorylation reaction of CaMKII substrates. Besides, phosphorylation activated CaMKII (p-CaMKII) can mediate the transmission of different signal transduction pathways, from the plasma membrane to the nucleus, by modulating downstream proteins to participate in the regulation of various physiological functions in organisms. P-CaMKII is also involved in the sensory processing of diversified noxious stimuli, including the transmission of noxious stimuli in the spinal cord. Hence, restraining Ca^{2+} influx is of crucial clinical significance for NP prevention and treatment.[4]

Kappa opioid receptor (KOR) is a subtype of opioid receptors and a member of the G protein-coupled receptor family, that is mainly distributed in central nervous tissues.[5] Its mRNA expression is found in rats' hippocampal dentate gyrus, hypothalamus and spinal cord.[6] Studies have demonstrated that hyperalgesia can be alleviated by injection of the KOR agonist U50488H into the rostral ventromedial medulla.[7] Moreover, the injection of the KOR agonist into the nucleus raphe magnus of rats can repress or enhance glutamatergic synaptic currents in the process of spinal pain transmission and attenuate the μ-opioid receptor agonist-induced analgesia.[8] It has been found that KOR agonist plays a crucial function in pain.[9] Meanwhile, cyclic AMP response element-binding protein (CREB) plays crucial roles in the modulation of neuropathic pain.[10] It has been reported that κ-opioid receptor agonist, U50488H, represses proptosis and improves synaptic plasticity by the Ca^{2+}/CaMKII/CREB signaling.[11]

In this study, we established a rat model of NP that was used to test U50488H through intrathecal injection, thermal withdrawal latency (TWL) and mechanical withdrawal threshold (MWT). The impacts of U50488H on calcium channel, inflammatory responses and oxidative stress injury of NP rats were observed. These experiments aimed at investigating the regulatory role of the Ca^{2+}/CaMKII/ CREB pathway to lay foundations for NP research and the development of therapeutic drugs.

Materials and Methods

Laboratory Animals and Grouping

A total of 40 SPF-grade male Sprague Dawley rats, weighing 220–260g each, were provided by the Department of Laboratory Animal Science of China Medical University. The rats were randomly divided into sham-operation group (Sham group, n=10), NP model group (NP group, n=10), NP + KOR agonist U50488H group (NU group, n=10) and NP + specific CaMKII antagonist (KN93) + U50488H group (NKU group, n=10). The rats in the NU group were administered with KOR agonist U50488H (1.5 mg/kg) via an intrathecal catheter 14 days after the SNI operation. Besides, the rats in the NKU group were continuously co-treated with U50488H and the specific CaMKII antagonist KN93 (intracisternal injection, 0.501 μg/kg) for 7 days after the SNI operation. The present study was reviewed and approved by the Laboratory Animal Welfare and Ethics Committee of Liaoning Province Tumor Hospital. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications 8023, revised 1978).

Establishment of Rat Model of NP

The NP model was established using the spared nerve injury (SNI) approach.[12] Specifically, the rats were anesthetized via intraperitoneal injection of pentobarbital sodium and fixed into supine position. Later, the operative region was depilated and disinfected and an incision (approximately 2 cm) was made on the skin at the superior margin of the hind limb. The muscles were bluntly dissected to expose the sciatic nerve trunk and the branches, including the tibial, the common peroneal and the sural nerves. Subsequently, the tibial and the common peroneal nerves were ligated and severed, and the small sural nerve preserved and protected from injury. Finally, the wound was sutured, layer by layer, and penicillin sodium was injected to prevent infection.

The rats in the NU group were administered with KOR agonist U50488H (1.5 mg/kg) via an intrathecal catheter 14 days after the SNI operation. Besides, the rats in the

NKU group were continuously co-treated with U50488H and the specific CaMKII antagonist KN93 for 7 days. Two hours following drugs administration, a pain behavior test was continually conducted to evaluate the changes in pain sensitivity. Finally, the rats were sacrificed, and the lumbar spinal cord (L_{4-6} segments) and dorsal root ganglia were obtained for subsequent experiments.

Pain Behavior Test

TWL and MWT were detected 1 day before operation, 7 and 14 days after operation and within 1 week after intrathecal administration. The rats were placed in transparent organic glass cages for TWL detection using a thermal hyperalgesia stimulator. When the left hind limb touched the glass plate, the left hind paw was irradiated with light sources at a certain focal length from the thermal hyperalgesia stimulator. The time from the start of irradiation to the retraction of the left hind limb was recorded by the stimulator built-in electronic stopwatch, which was taken as the numerical value of TWL.

A dynamic plantar aesthesiometer was used to determine the MWT of the area innervated by sural nerves of the rats' lateral left hind paws. Specifically, the plantar skin of the right hind limb of the rats was vertically stimulated from the bottom, and the upward force was gradually increased until an acute retraction of the hind paw was observed, which was deemed as a positive behavior. Finally, the MWT was recorded.[13]

Hematoxylin–Eosin (HE) Staining

Formalin-fixed spinal cord tissues were sequentially dehydrated in 70%, 80%, 90%, 95% and 100% ethanol, transparentized in xylene and paraffin-embedded into blocks. The tissue blocks were sliced into 4 μm-thick sections and subjected to the H&E staining. Finally, the sections were mounted in neutral balsam and photographed under a light microscope (CX33, Olympus Corporation, Japan).

Nissl Staining

The spinal cord paraffin sections were deparaffinized with xylene, soaked in Nissl staining solution for 10 min, washed in water, separately placed in 70%, 90% and 100% ethanol for 1 min, transparentized with xylene, mounted with neutral resin and photographed under a light microscope.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL)

The sections of dorsal root ganglia were processed with 50 μL of TUNEL reaction solution and incubated at 37°C in the dark for 60 min. An amount of 50 μL streptavidin-horseradish peroxidase (HRP) working solution was added onto the sections for incubation in a lightproof box for 30 min. Finally, the nuclei were subjected to fluorescence staining using a 4′,6-diamidino-2-phenylindole (DAPI) solution, and the sections were mounted in an anti-fade fluorescence mounting medium and photographed under a fluorescence microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

Inflammatory factors [interleukin-1 beta (IL-1β) (CSB-E08055r, CUSABIO), IL-6 (SEA079Ra, USCN), tumor necrosis factor-alpha (TNF-α) (SEA133Si, USCN)], oxidative stress indexes [superoxide dismutase (SOD) (SES134Hu, USCN), malondialdehyde (MDA) (CEA597Ge, USCN), glutathione peroxidase (GSH-Px) (CEA294Ge, USCN)] and glutamate (Glu) (CES122Ge, USCN) were measured in the dorsal root ganglia using ELISA kits and following the manufacturer's instructions. An amount of 100 μL standard substances, at different concentrations, and 100 μL of diluted test samples were added to the plate for a 2 h of incubation at 37°C, then the plate was washed in PBST, and 100 μL of HRP-labeled secondary antibodies were added into each well and incubated at 37°C for 30 min. Following plate washing, 50 μL of developer A and 50 μL of developer B were added for color development in the dark for 15 min. Subsequently, 50 μL of stop buffer was added, and the OD value at 450 nm was measured using a microplate reader (EXL800, USA). Finally, standard curves were plotted, and the concentrations of corresponding samples were calculated based on a curvilinear equation.

Immunofluorescence (IF) Assay

The paraffin-embedded sections of the dorsal root ganglia were deparaffinized, rehydrated, soaked in 3% hydrogen peroxide solution and washed with phosphate-buffered saline (PBS). Then, the sections were immersed in 0.1 M sodium citrate solution for antigen retrieval, sealed in goat serum and incubated at 37°C for 30 min. Next, a Ca^{2+} fluorescent probe,

a reactive oxygen species (ROS) probe and antibodies against CGRP and NMDAR were added separately for incubation at 4°C overnight. The sections were washed with PBS and then incubated with fluorescence labeled secondary antibodies at 37°C for 30 min. Finally, the nuclei were stained with DAPI and incubated at room temperature for 10 min, followed by washing with PBS. The sections were mounted with an anti-fade fluorescence mounting medium and photographed under a fluorescence microscope.

Western Blotting

The cryopreserved tissues of the dorsal root ganglia were lysed using a protease lysis buffer and centrifuged to obtain supernatants. Following protein concentration measurement using the BCA method, the proteins were subjected to SDS-PAGE, transferred to a membrane, sealed in skim milk for 2 h and incubated with antibodies against nuclear factor-kappa B (NF-κB) (Abcam, USA, 1:1000), CaMKII (Abcam, USA, 1:1000), p-CaMKII (Abcam, USA, 1:1000), CREB (Abcam, USA, 1:1000), p-CREB (Abcam, USA, 1:1000) and GAPDH (Abcam, USA, 1:1000) at 4°C overnight. This step was followed by 3 times membrane washing and incubation with an HRP-labeled goat anti-rabbit IgG antibody at 37°C for 1 h. Finally, the color of the proteins was developed using an ECL kit and a gel imaging system. The absorbance was analyzed by the ImageJ software.[13]

Statistical Analysis

The SPSS 20.0 software was used for statistical analysis. The experimental data were presented as mean ± standard deviation (x ± s), and one-way analysis of variance was used to compare data between groups. Statistic comparison between two groups or multiple groups were evaluated by Student's *t* test or one-way ANOVA. Two-way ANOVA was used for Figure 9C and D. $P < 0.05$ was considered statistically significant.

Results

The KOR Agonist Improved the MWT and TWL of NP Rats

The NP rat model was established through the SNI method and the results indicated that MWT was remarkably reduced (Figure 1A), and TWL notably shortened at 7 and 14 days after modeling (Figure 1B), suggesting that the NP model was successfully established. Following U50488H administration, the results of constant detection showed a gradual restriction of MWT (Figure 1C) and a shortening of TWL (Figure 1D). The results also showed that their levels were evidently higher than those in the NP group ($p < 0.05$).

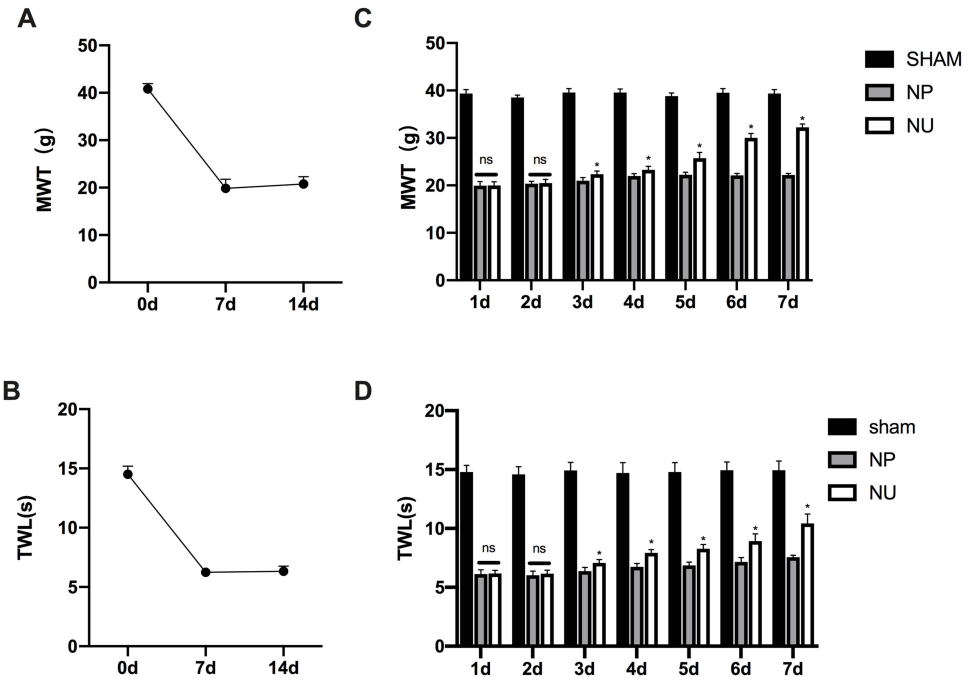

Figure 1 The KOR agonist improved the MWT and TWL of NP rats. (**A**) Reflex Threshold (MWT) in NP rats at 7d and 14d post-modeling; (**B**) Heat Shrink Foot Latent Period (TWL) in NP rats at 7d and 14d post-modeling; (**C**) Reflex Threshold (MWT) in rats with KOR agonist administration in the following 7 days post-administration; (**D**) Heat Shrink Foot Latent Period (TWL) in rats with KOR agonist administration in the following 7 days post-administration; *$p < 0.05$.

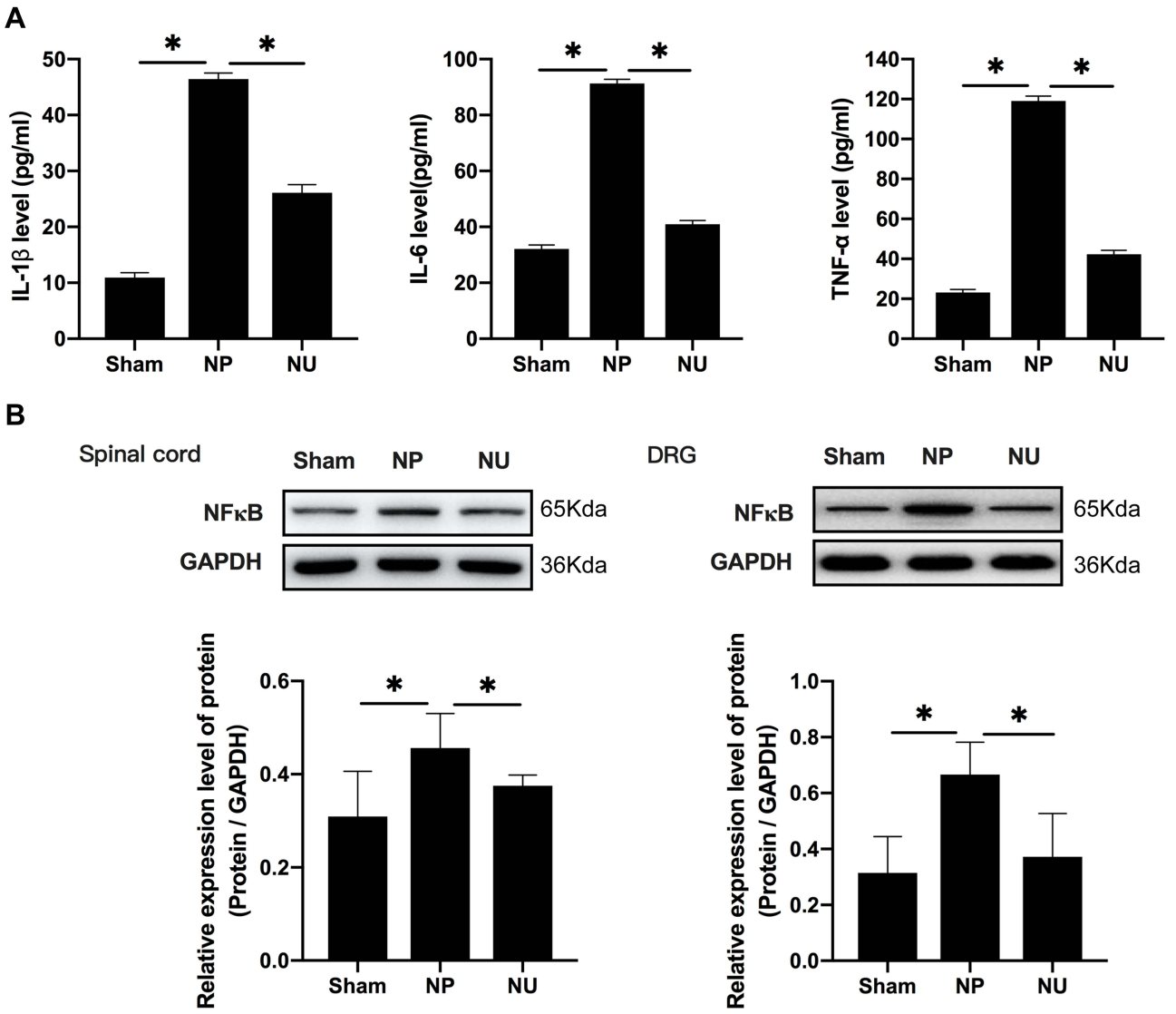

Figure 2 The KOR agonist inhibited NF-κB expression and relieved inflammatory responses in NP rats. (**A**) Inflammatory factors (IL-1β, IL-6 and TNF-α) detected by ELISA; (**B**) NF-κB expression level in rats' spinal cords detected by Western blotting; NF-κB expression level in rats' dorsal root ganglia detected by Western blotting; *$p < 0.05$.

The KOR Agonist Inhibited NF-κB Expression and Relieved Inflammatory Responses in NP Rats

Neuroinflammation is a major factor for NP; therefore, the inflammatory factors in the serum of each group of rats were examined first. The results showed an increase in IL-1β, IL-6 and TNF-α serum concentrations in the NP group (vs Sham group, $p < 0.05$), while the in vivo inflammatory responses in the NU group were repressed on day 7 after administration (Figure 2A). Furthermore, the expression of NF-κB (p65) in the spinal cord (Figure 2B) and dorsal root ganglia was tested, and the results indicated that its expression declined in the NU group (vs NP group, $p < 0.05$). These results demonstrate that the KOR agonist can inhibit NF-κB expression and relieve inflammatory responses in NP rats.

The KOR Agonist Lowered ROS Expression and Mitigated Oxidative Stress Injury in NP Rats

Activated inflammatory responses can facilitate oxidative stress injury and ROS increase, which association with neurodegenerative disorders has been proven. In this study, ELISA was first conducted to measure serum contents in SOD, MDA and GSH-Px, and the results showed that the KOR agonist could enhance the release of SOD and suppress the secretion of MDA and GSH-Px (vs NP group, $p < 0.05$) (Figure 3A). Moreover, the results of the

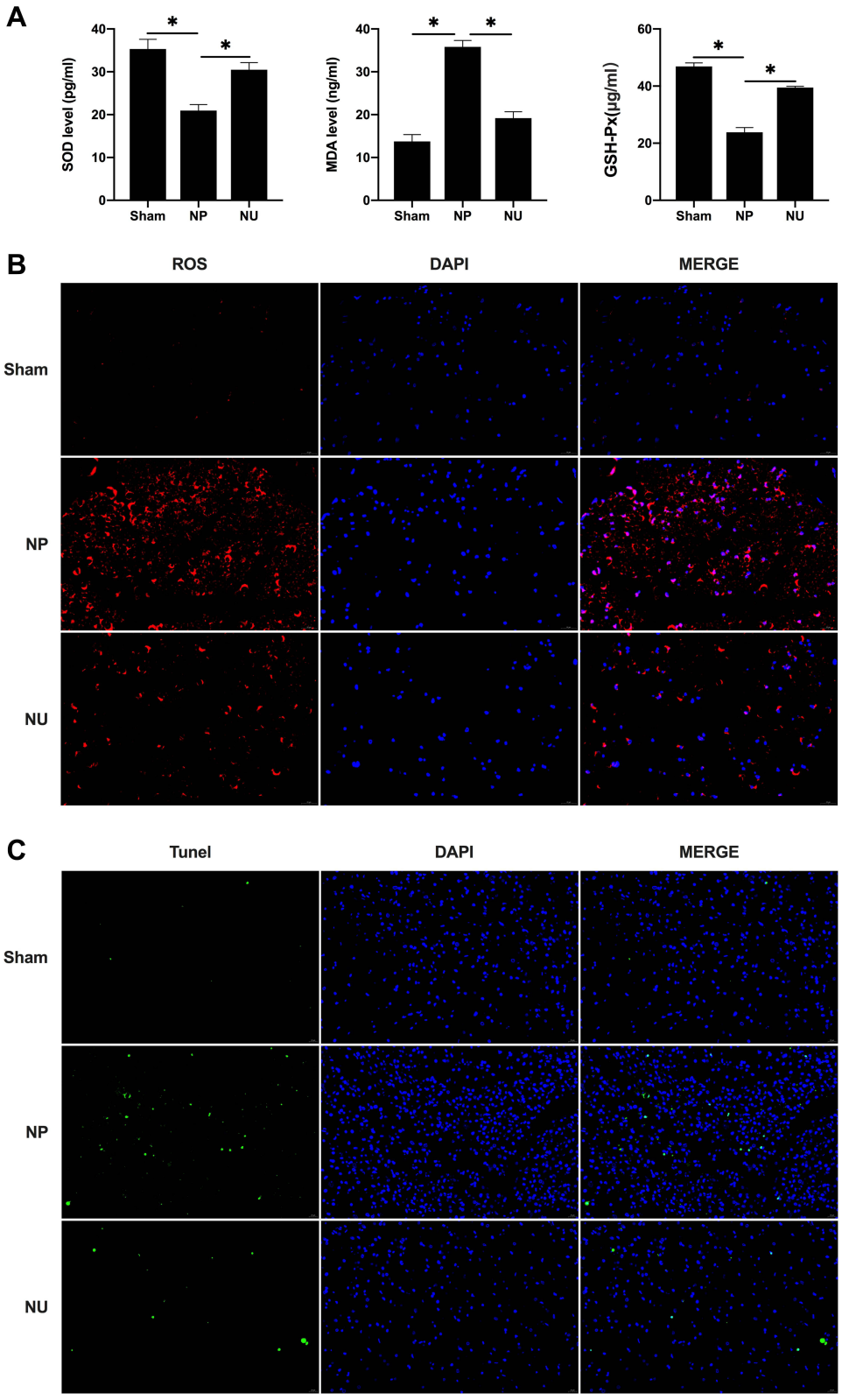

Figure 3 The KOR agonist lowered ROS expression and mitigated oxidative stress injury in NP rats. (**A**) Oxidative stress factors (SOD, MDA and GSH-Px) detected by ELISA; (**B**) The expression of ROS in rats' dorsal root ganglia detected by IF (scale bar = 20μm); (**C**) The apoptosis rate detected by TUNEL staining (scale bar = 20μm); *p < 0.05.

IF assay, that assessed ROS expression in the dorsal root ganglia using red fluorescence, demonstrated an increased level of ROS in the NP group compared to the NU group (Figure 3B). This result suggests that the KOR agonist can inhibit the release of ROS. In addition, the excessive oxidative stress injury in NP rats caused cell apoptosis in the dorsal root ganglia and a notable decrease in the apoptosis rate in the NU group compared to that in the NP group (Figure 3C). These results imply that the KOR agonist can down-regulate ROS expression and mitigate oxidative stress injury in NP rats.

The KOR Agonist Down-Regulated Neuropeptide CGRP and Ameliorated Neuronal Injury

After the incidence of neural injury, the inflammatory factors that are released by immune cells, are capable of interacting with neurons and glial cells that release CGRP and NMDAR. The ligation of the sciatic nerve could induce neuronal injury in the spinal cord of NP rats (Figure 4A), with a reduction in the number of neurons (Figure 4B). Besides, the expressions of CGRP and NMDAR in the dorsal root ganglia increased (Figure 5), but the KOR agonist could alleviate spinal cord neuronal injury and suppress the release of CGRP and NMDAR in the dorsal root ganglia.

The KOR Agonist Suppressed the Ca^{2+}/CaMKII/CREB Pathway in NP Rats

Following CGRP and NMDAR overactivation, Ca^{2+} cell membrane permeability increases, resulting in cellular calcium overload. In the NP group, Ca^{2+} and Glu contents in the dorsal root ganglia increased (Figure 6A and B), resulting in Ca^{2+} binding to CaMKII, which promotes the CaMKII and CREB phosphorylation (Figure 6C). However, following the KOR agonist administration, Ca^{2+} influx, the Glu content and CaMKII and CREB phosphorylation decreased.

The KOR Agonist Alleviated NP in Rats Through the Ca^{2+}/CaMKII/CREB Pathway

To explore the regulatory effect of the Ca^{2+}/CaMKII/CREB pathway on NP rats, U50488H and a specific CaMKII antagonist (KN93) were simultaneously administered. The results showed an inhibition of KOR agonist-mediated reduction of Ca^{2+} influx (Figure 7) and an increase of CGRP and NMDAR expression in the dorsal root ganglia (Figure 8). Moreover, the number of neurons in the spinal cord was reduced compared to those in the NU group (Figure 9A) and the apoptotic rate increased in the dorsal root ganglia (Figure 9B). Finally, MWT was lower and TWL shorter on day 3 and 7 in the NPU group compared to the NU group ($p < 0.05$) (Figure 9C and D). Taken together, these results imply that the CaMKII antagonist can repress the NP ameliorative effect of the KOR agonist.

Discussion

NP is a result of complex interactions between peripheral and central nervous systems.[14] The pathophysiological changes in primary sensory neurons and the abnormalities of signal processing in the central nervous system constitute the basic mechanisms of NP. Surgery, viral infection, chemotherapy or cancer can lead to NP, which can progress into chronic pain, that greatly affects patients' normal life.[1,15–17] Therefore, it is a medical problem that needs to be urgently solved. In this experiment, the NP rat model was generated using the SNI method.[12] The rats manifested low spirit, reduced activities and prominent decreases in TWL and MWT, which are common manifestations also observed in NP patients, including depression, anxiety and apathy.[18] Following U50488H intrathecal injection, the TWL was prolonged, the MWT increased and the pain relieved. These effects correlated with the activation of the Ca^{2+}/CaMKII/CREB pathway.

U50488H is a highly selective KOR agonist, whose selective binding and activation properties, are 1300 times that of the μ-opioid receptor and 12,000 times that of the δ-opioid receptor.[8] Currently, U50488H is widely applied in research on the physiological action of KOR. It has been shown that the intrathecal injection of U50488H can remarkably improve cardiac function and repress myocardial fibrosis of rats with myocardial ischemia/reperfusion injury, whose mechanism correlated with the regulation of CGRP and ET release. Moreover, U50488H exerts its neuroprotective effects by specifically activating KOR on hippocampal neurons. According to an experiment on spinal cord injury (SCI) in rats, the KOR agonist cannot enhance functional recovery after SCI. On the

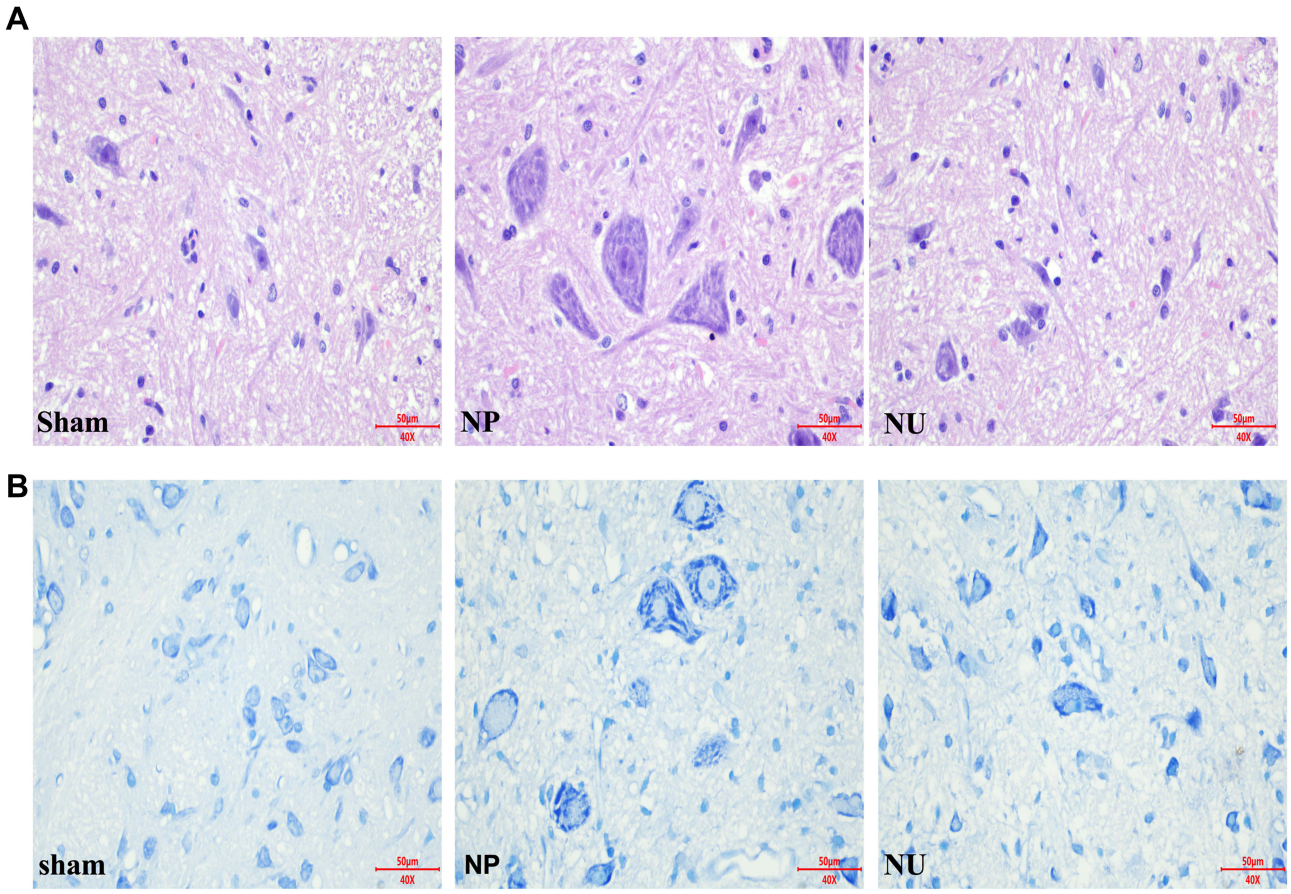

Figure 4 The KOR agonist down-regulated neuropeptide CGRP and ameliorated neuronal injury. (**A**) HE staining (scale bar = 50μm); (**B**) Nissl staining (scale bar = 50μm).

contrary, the selective KOR agonist U50488H cannot induce dysfunction, but it is able to improve the recovery of the spinal cord blood flow and function.[19] In the present study, the NP rats were treated with U50488H, and the values of TWL and MWT were increased from the 3rd day of drug administration. Furthermore, inflammatory and oxidative stress factors were assessed to investigate its mechanism of action. The results indicated that U50488H had an inhibitory effect on neuroinflammation and that the expression of NF-κB was decreased in both dorsal root ganglia and spinal cord. It is well known that NF-κB is a crucial player during pain, and its expression in the spinal dorsal horns and dorsal root ganglia can be activated by neuropathic stimuli, such as chronic constriction injury of sciatic nerve, sciatic nerve transection and sciatic nerve injury. U50488H could alleviate the clinical manifestations of NP through NF-κB repression.[20]

The integrated network formed by immune cells, glial cells and neurons plays an important role in the occurrence and development of NP.[18] Inflammatory factors that are released by immune cells can interact with neurons and glial cells can release CGRP and NMDAR.[21] In a study on cerebral ischemia/reperfusion injury, it was shown that the injection of μ-opioid receptor, δ-opioid receptor and KOR into the lateral ventricle can concomitantly protect the brain and weaken the release of CGRP.[22] In this study, the NP rats manifested high expression levels of CGRP and NMDAR in the dorsal root ganglia and a reduced number of spinal cord neurons. The KOR agonist could alleviate neuronal damage in the spinal cord and suppress the release of CGRP and NMDAR in the dorsal root ganglia, suggesting that the NP protective effect of U50488H is associated with the inhibition of Ca^{2+} overload.

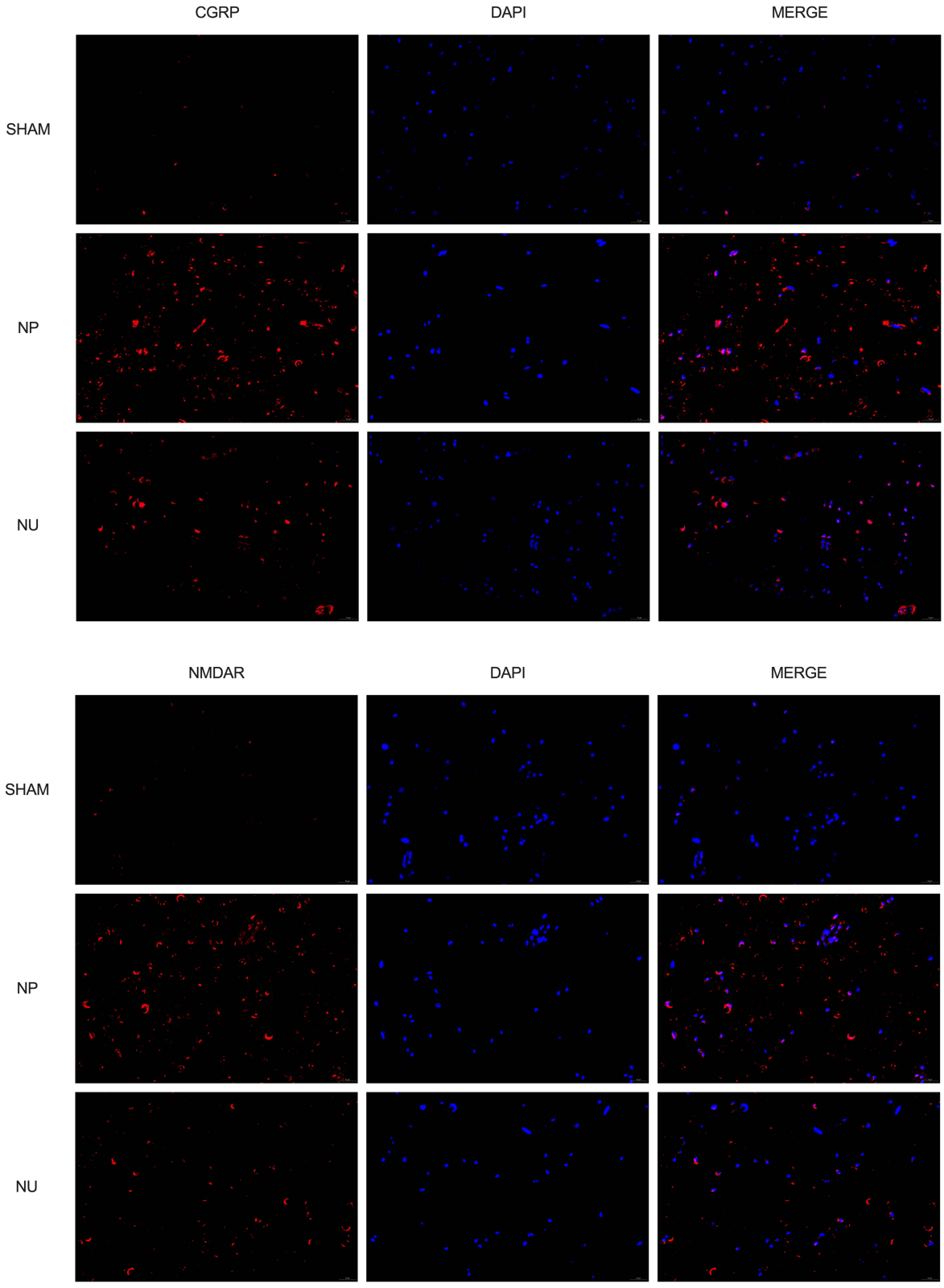

Figure 5 The expression of CGRP and NMDAR affected by KOR agonist in rats' dorsal root ganglia detected by IF (scale bar = 20μm).

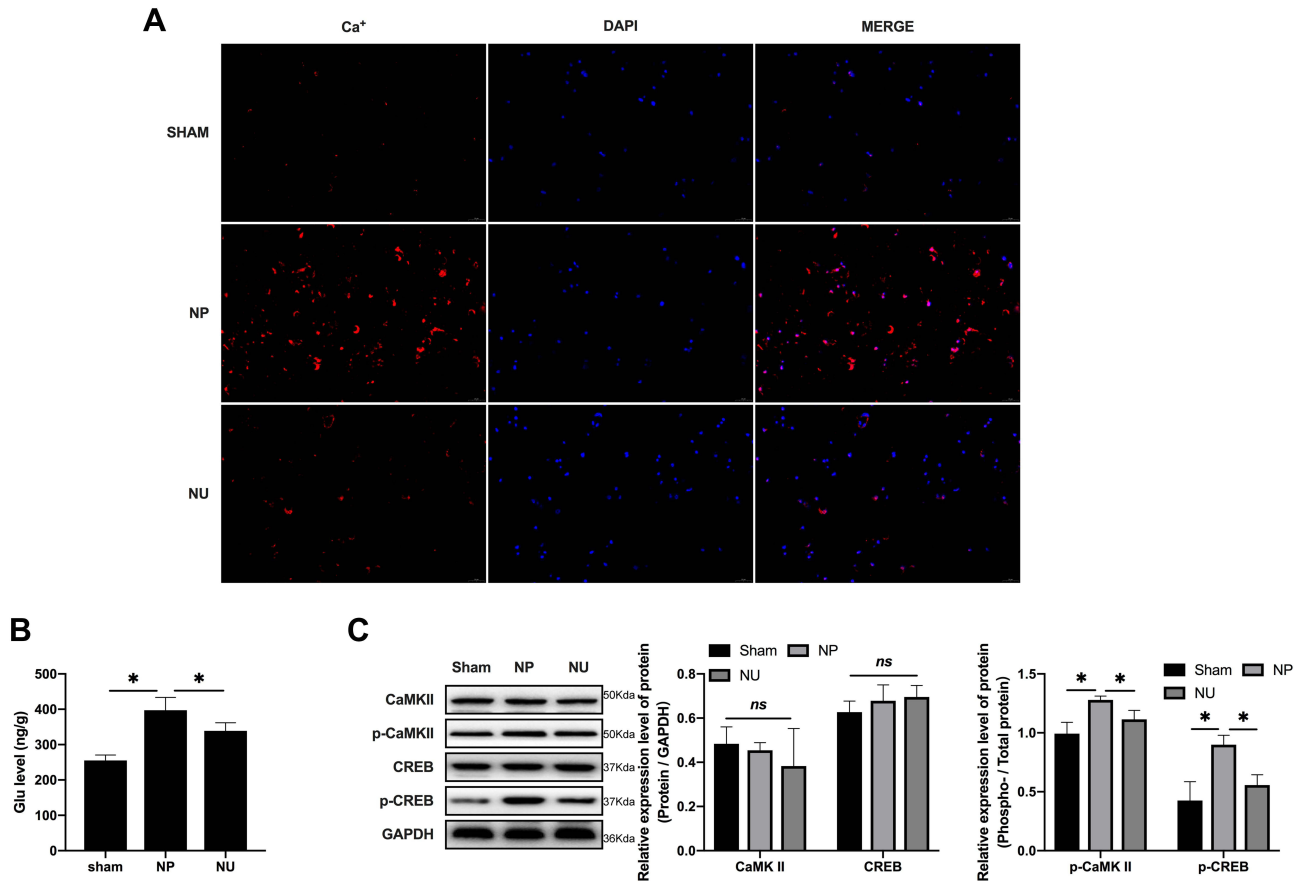

Figure 6 The KOR agonist suppressed the Ca^{2+}/CaMKII/CREB pathway in NP rats. (**A**) Cellular calcium level in dorsal root ganglia detected by IF (scale bar = 20μm); (**B**) Glutamate level in rats' dorsal root ganglia detected by ELISA; (**C**) The protein expression level of CaMKII, CREB and related phosphorylated protein detected by Western blotting; *$p < 0.05$.

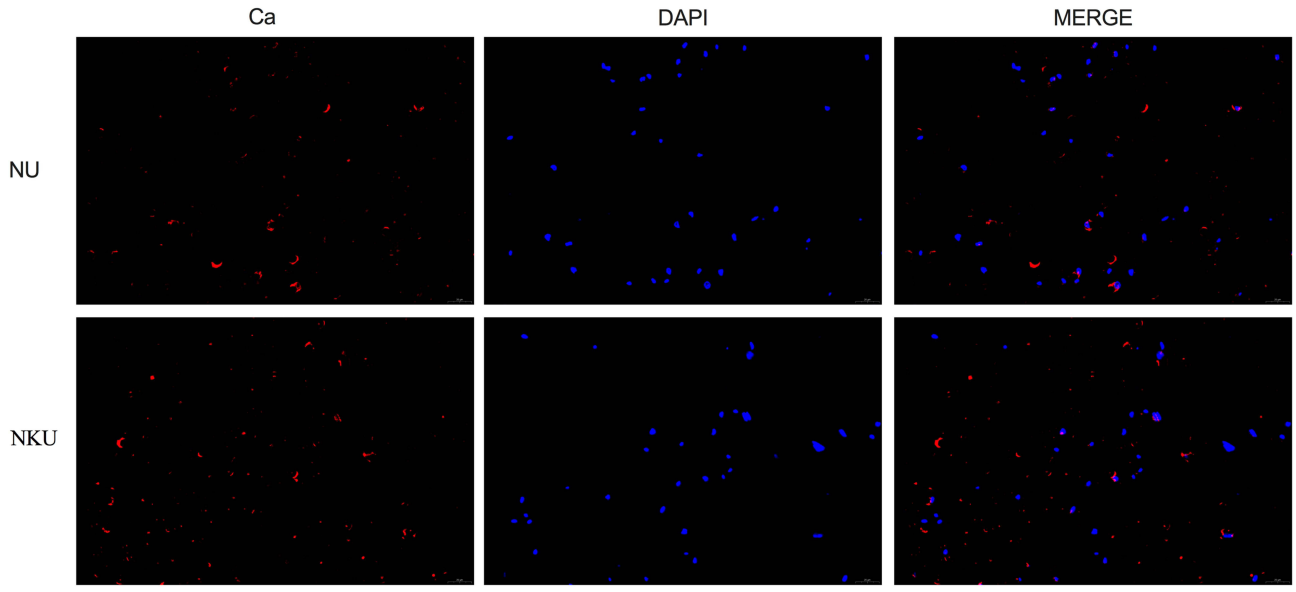

Figure 7 The KOR agonist alleviated NP in rats through the Ca^{2+}/CaMKII/CREB pathway. Cellular calcium level detected by IF (scale bar = 20μm).

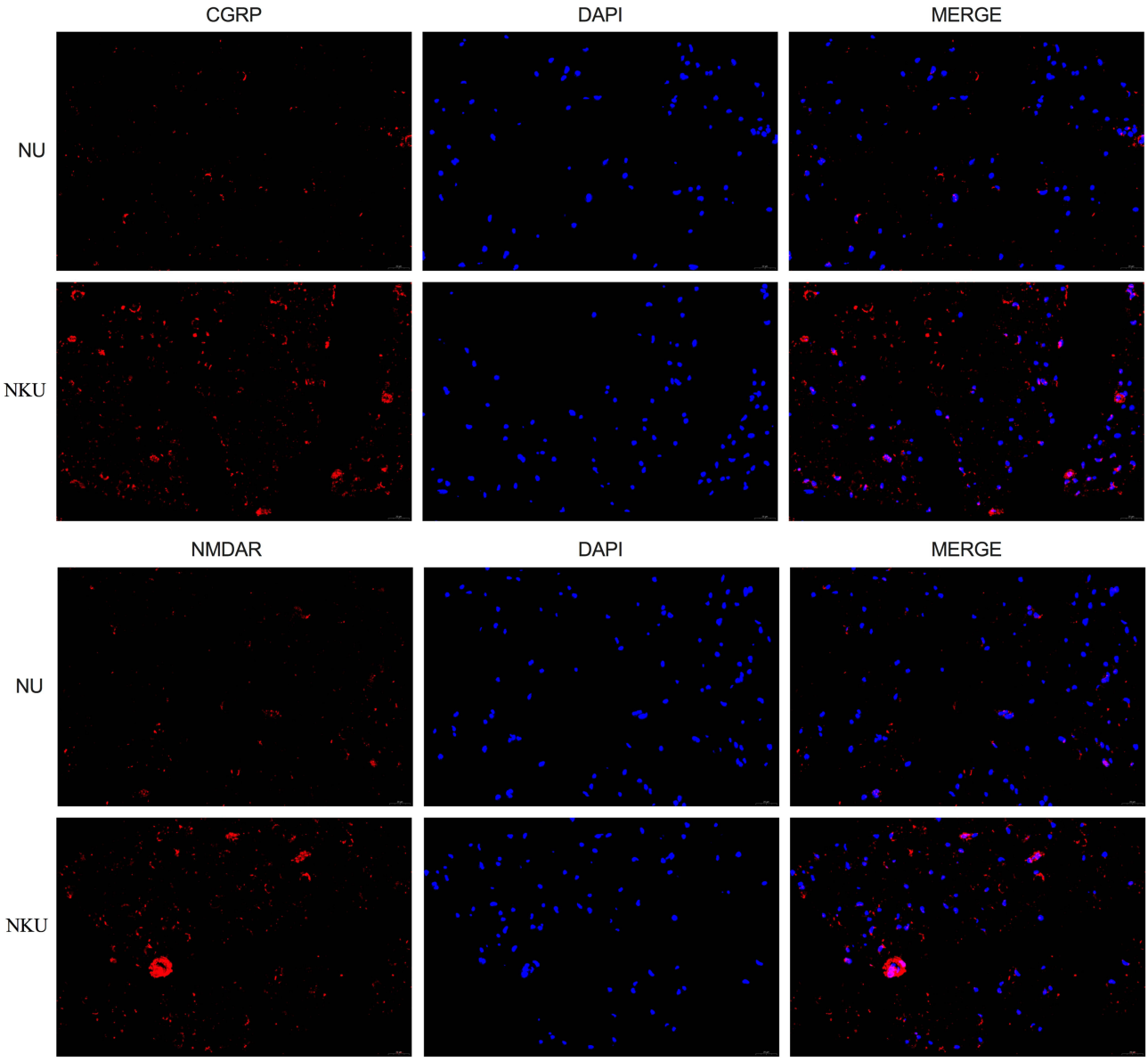

Figure 8 The expression of CGRP and NMDAR combinedly affected by specific CaMKII antagonist (KN93) + U50488H group in rats' dorsal root ganglia detected by IF (scale bar = 20μm).

NMDAR overactivation can increase Ca^{2+} permeability of the cell membrane and stimulate a massive Ca^{2+} influx that causes intracellular calcium overload.[23] Ca^{2+} binding to CaMKII triggers the phosphorylation of CaMKII substrates and facilitates its own phosphorylation.[24] Furthermore, the activated CaMKII can phosphorylate CREB, conjugate with cAMP-responsive elements on the target genes and recruit RNA polymerase II to compose transcription complexes, which modulate target genes' transcription. Previous studies demonstrated that CREB can regulate the transcription of multiple pain genes, such as c-Fos, c-Jun, NK-1, COX-2 and BDNF. These changes in CREB-mediated transcription are involved in the development and maintenance of pain central sensitization, which is the neurobiological basis of transformation from acute lesion into chronic pain.[25] Based on the study results, Ca^{2+} level in the dorsal root ganglia was markedly elevated in NP rats. This was corroborated by the increased levels of p-CaMKII and p-CREB, assessed by Western blotting. Besides,

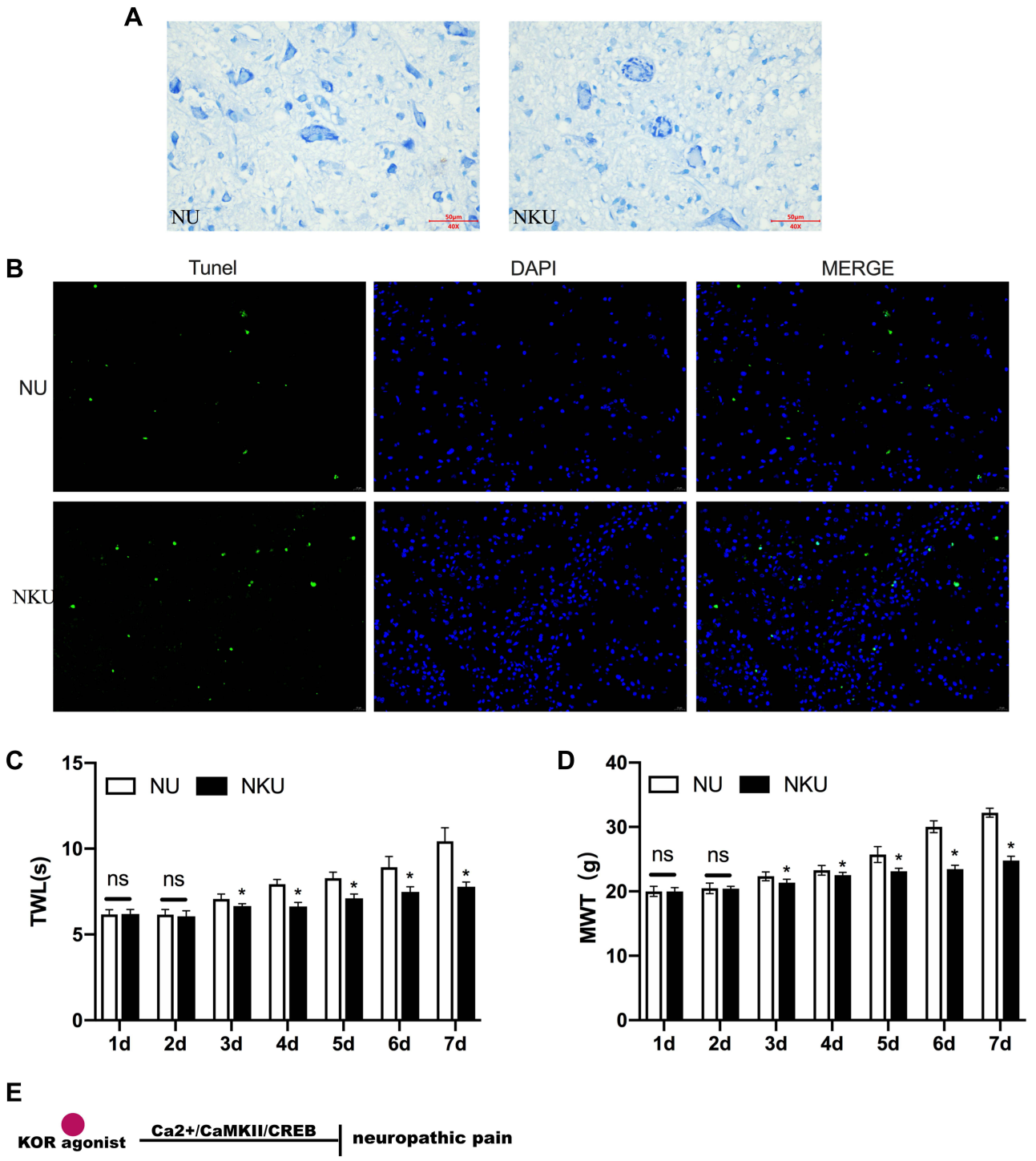

Figure 9 (**A**) Nissl staining (scale bar = 50μm); (**B**) The apoptotic rate detected by TUNEL staining (scale bar = 20μm); (**C**) Heat Shrink Foot Latent Period (TWL) in rats with the administration of CaMKII antagonist; (**D**) Reflex Threshold (MWT) in rats with the administration of CaMKII antagonist; (**E**) A model of this study was shown. *p < 0.05.

U50488H could restrain Ca^{2+} influx and down-regulate p-CaMKII and p-CREB levels. After administration of the CaMKII antagonist, the protective effect of U50488H on NP was attenuated, but no effect was observed with regard to inflammatory responses and oxidative stress injury. It was presumed that the KOR agonist can alleviate NP in rats through the Ca^{2+}/CaMKII/CREB pathway (Figure 9E).

Funding

This work was supported by the Liaoning Natural Fund Project (grant number 20180550218).

Disclosure

The authors report no conflicts of interest in this work.

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