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ORIGINAL RESEARCH

# Increased cerebral nuclear factor kappa B in a complex regional pain syndrome rat model: possible relationship between peripheral injury and the brain

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**Purpose:** Complex regional pain syndrome (CRPS) is a rare but refractory pain disorder. Recent advanced information retrieval studies using text-mining and network analysis have suggested nuclear factor kappa B (NFκB) as a possible central mediator of CRPS. The brain is also known to play important roles in CRPS. The aim of this study was to evaluate changes in cerebral NFκB in rats with CRPS.

**Materials and methods:** The chronic post-ischemia perfusion (CPIP) model was used as the CRPS animal model. O-rings were applied to the left hind paws of the rats. The rats were categorized into three groups according to the results of behavioral tests: the CPIP-positive (A) group, the CPIP-negative (B) group, and the control (C) group. Three weeks after the CPIP procedure, the right cerebrums of the animals were harvested to measure NFκB levels using an ELISA.

**Results:** Animals in group A had significantly decreased mechanical pain thresholds ($P<0.01$) and significantly increased cerebral NFκB when compared to those in groups B and C ($P=0.024$).

**Conclusion:** This finding indicates that peripheral injury increases cerebral NFκB levels and implies that minor peripheral injury can lead to the activation of pain-related cerebral processes in CRPS.

**Keywords:** cerebrum, complex regional pain syndrome, enzyme-linked immune-sorbent assay, nuclear factor kappa B, pain

## Introduction

Complex regional pain syndrome (CRPS) is a chronic, debilitating, and difficult-to-treat painful disease that occurs after surgery or minor trauma. Many possible mechanisms, including peripheral and central sensitization, sympatho-afferent coupling, brain changes, and genetic and psychological factors, are implicated in the development of this disorder.[1] Because there have been many cases in which treatments targeted to peripheral nerves or tissues have not been satisfactory in clinical practice, several efforts have been made to evaluate the role of the brain in patients with CRPS. There is increasing evidence that the central nervous system is involved in the development and maintenance of CRPS. For example, patients with CRPS have distorted body images and fail to recognize the size of the affected limb.[2] A mirror image study has shown that mechanical stimulation of the virtual (unaffected) limb results in allodynia, which implies that changes in the brain contribute to the maintenance of painful symptoms of CRPS.[3] Moreover, several studies have shown that changes occur in the brain in patients with CRPS[4,5] and in CRPS animal models.[6]

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The molecular mechanisms and key mediators of CRPS pathogenesis have been investigated extensively. As a result, many molecular pathways have been proposed to underlie CRPS development. Interestingly, a team of researchers with various backgrounds (physicians, bioinformaticians, and text-mining experts) has used an advanced information retrieval method consisting of text-mining and network analysis to investigate a network of CRPS-related mechanisms and concepts. This team has identified nuclear factor kappa B (NFκB) as a possible central mediator in both the initiation and progression of CRPS.[7] In the abovementioned study, NFκB had a strong connectivity with other mediators and was shown to be involved in many physiological processes in CRPS.

NFκB is found in almost all animal cell types and is involved in cellular responses to stimuli, such as stress, cytokines, free radicals, and bacterial or viral antigens.[8,9] It is a pivotal mediator in various physiological processes[10] and a ubiquitous transcription factor in the initiation of diseases.[11] Aberrant regulation of NFκB is known to be related to malignancy,[12,13] inflammatory and autoimmune diseases,[14] and improper immune development.[15] Moreover, NFκB has also been implicated in processes of synaptic plasticity[16,17] and memory,[18–20] which are related to chronic pain. Many studies have demonstrated NFκB to be an essential transcription factor mediating the actions of neuropeptides involved in CRPS.[21–23] Although the importance of the role of NFκB has been investigated in neuropathic pain, most studies have focused on the peripheral nervous system or on the spinal cord and especially on the dorsal root ganglion.[24–26] Hence, no information is available on NFκB changes in the brain in the context of CRPS. Therefore, this study was conducted to investigate whether changes in cerebral NFκB occur using a CRPS animal model.

# Materials and methods

## Animals

The authors complied with the Guide for the Care and Use of Laboratory Animals of the National Research Council and the ethical guidelines for animal research by Seoul National University Bundang Hospital. This study was approved by the Institutional Animal Care and Use Committee (IACUC number: BA 1008-068/052-03). Male Sprague Dawley rats weighing 200–250 g were allowed free access to food and water and were housed individually in cages under a 12-hour night/day cycle (lights on/off at 7 am/7 pm) at a constant temperature of 20–22°C and a humidity level of 55–60%. The animals were acclimated for at least 1 week prior to the generation of the CRPS model.

## Generation of CRPS animal model

A chronic post-ischemia perfusion (CPIP) rat model was used in this study, which was generated according to the previous methods.[27] After the induction of anesthesia with isoflurane, a tight-fitting Nitrile 70 Durometer O-ring® (O-ring West Inc., Lynnwood, WA, USA) with a 5.5 mm internal diameter was applied to the left hind limb of each rat just proximal to the left ankle joint for 3 hours. Three hours later, the O-ring was cut off from the anesthetized rat, allowing reperfusion of the hind limb. The animals in the control group received anesthesia in the same manner, but the O-ring was not placed around the hind limb.

## Behavioral tests

Behavioral tests were performed as previously described.[28,29] All behavioral tests were performed between 9 am and 3 pm. To assess the mechanical threshold (MT), each rat was placed in an individual plastic cage with a wire mesh bottom. After a 20-minute acclimation period, calibrated von Frey filaments (Stoelting Co.; Wood Dale, IL, USA) with logarithmically increasing stiffnesses of 0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.10 g were sequentially applied to the mid-plantar surface of the left hind paw. Starting with the 2.00 g filament, rapid withdrawal or flinching was interpreted as a positive response, in which case the next lighter filament was applied; a negative response led to the application of the next heavier filament. The MT was assessed using an up–down statistical method.[30] The change in the MT (CMT, %) was calculated using the following equation:

$$CMT\ (\%) = (MT_{post} - MT_{pre})/MT_{pre} \times 100$$

The MT was examined during the post-reperfusion period at the following time points: 1 hour, 4 hours, 24 hours, 48 hours, 7 days, and 21 days.

The findings from the neurobehavioral tests on day 21 were used to classify the animals into three groups: rats whose CMT was decreased by 50% or more after the CPIP procedure were classified as the CPIP-positive group (A), and the animals whose CMT was decreased by <50% were classified as the CPIP-negative group (B). The MTs of the animals in the control group (C) were also examined.

## ELISA to assess NFκB levels

An ELISA kit (USCN Life Science Inc., Wuhan, China) was used to determine the levels of NFκB in rat brain samples (n=5, in each group). After saline infusion into the left heart

ventricle of each rat, the brain was harvested and the right cerebrum was separated from the whole brain. One hundred and twenty milligrams of minced tissue was homogenized using 500 µL of PBS (20 mM, pH 7.0) and centrifuged for 5 minutes at 5,000 × *g*. Supernatants were diluted with 20 mM PBS to a final dilution of 1:3 (volume:volume). The assay was performed according to the manufacturer's instructions. Standards/sample supernatants were incubated with NFκB antibody in pre-coated 96-well microplates. A biotin-conjugated antibody specific for NFκB was added to the microplates. The samples were incubated for 1 hour at 37°C. Plates were then washed five times with wash buffer and incubated with avidin-conjugated horseradish peroxidase for 30 minutes at 37°C. Tetramethylbenzidine was added to the samples after seven washes, and the plates were incubated for 20 minutes at 37°C. Finally, sulfuric acid was added to the samples to stop the reaction. The microplates were analyzed using a spectrophotometer (wavelength: 450 nm). Samples were analyzed in duplicate, and protein levels were interpolated according to the established standard curve (detection range: 0.312–20 ng/mL).

## Statistical analyses

Data are reported as median and 75% CIs. Kruskal–Wallis test followed by Dunn's post hoc analysis was used to compare the NFκB expression levels and the paw withdrawal thresholds in the three groups at each time point. All statistical analyses were performed using SPSS version 19.0 (IBM Corporation, Armonk, NY, USA). *P*-values of <0.05 were considered statistically significant.

# Results

Fifteen animals (n=5 per group) were included in this study. There were no differences in the mechanical withdrawal threshold among the groups before the CPIP procedure. However, animals in group A exhibited a significant decrease in the mechanical withdrawal threshold when compared to those in groups B and C, 1 hour after the CPIP procedure, and it continued to decrease up to day 21 ($P<0.01$; Figure 1). The expression of NFκB was significantly increased in group A when compared to groups B and C ($P=0.024$; Figure 2). There was no significant difference in the cerebral NFκB level between groups B and C.

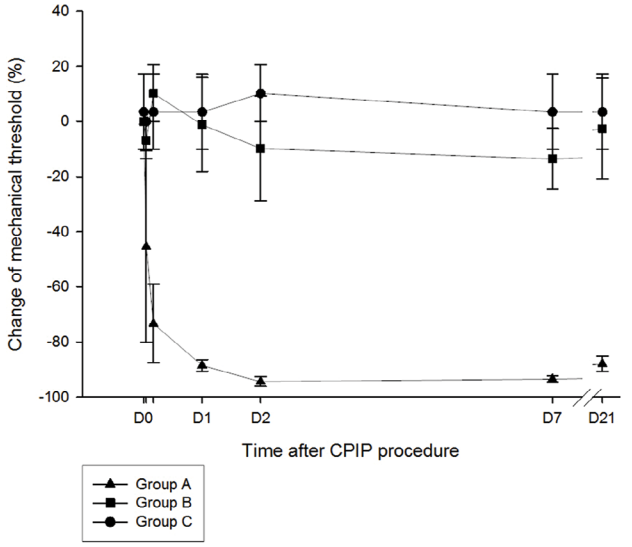


**Figure 1** The change in mechanical threshold (%) after O-ring application.
**Notes:** The mechanical threshold was examined during the post-reperfusion period at the following time points: 1 hour, 4 hours, 24 hours, 48 hours, 7 days, and 21 days. Animals in group A exhibited a significant decrease in the mechanical threshold when compared to those in groups B and C (n=5 in each group, $P<0.01$). Group A, CPIP-positive group; group B, CPIP-negative group; group C, control group. Error bars indicate standard errors.
**Abbreviations:** CPIP, chronic post-ischemia perfusion; D, day.

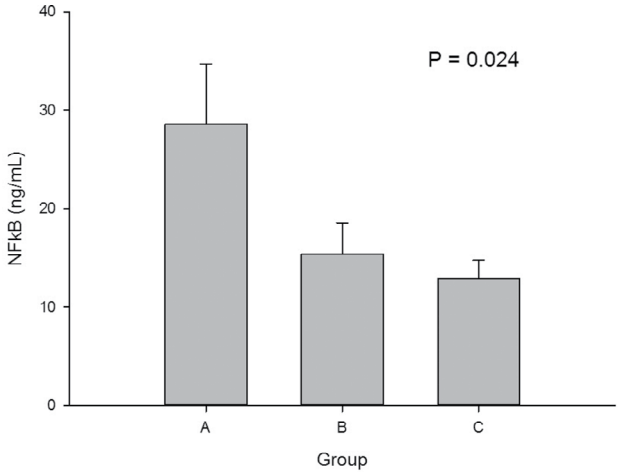


**Figure 2** NFκB in the rat cerebrum after O-ring application.
**Notes:** Animals in group A exhibited a significant increase in NFκB levels when compared to those in groups B and C (n=5 in each group, $P=0.024$). Data are described as median and 75th percentile. Group A, CPIP-positive group; group B, CPIP-negative group; group C, control group. Error bars indicate standard errors.
**Abbreviations:** CPIP, chronic post-ischemia perfusion; NFκB, nuclear factor kappa B.

# Discussion

In this study, we report that the cerebral expression level of NFκB is increased in the CPIP animal model. This finding implies that minor peripheral injury can affect the cerebrum. Considering that minor peripheral injury is a well-known cause of CRPS and that NFκB plays many pivotal physiological functions, this finding has implications for the investigation of cerebral changes in CRPS.

NFκB is a protein complex that regulates many cellular signaling pathways in response to a wide variety of stimuli. It can regulate the transcription of genes, such as cytokines, pro-inflammatory enzymes, and pro-inflammatory transcription factors, which have essential roles in inflammation and the immune response.[31] Activation of NFκB is related to various pain conditions, such as rheumatoid arthritis,[32] migraine,[33] and neuropathic pain.[34,35] It has also been reported that injection of an NFκB decoy that prevents activated NFκB from binding to a consensus DNA fragment and initiating transcription can suppress cytokine expression and neuropathic pain.[36,37] NFκB has diverse functions in the nervous system and is involved in the pathogenesis of neurodegenerative disorders and learning and memory.[20,38] Long-term potentiation and synaptic plasticity, which are known to be related to abnormal pain perception,[39] are mediated by NFκB activation.[16,20,40] Although NFκB is ubiquitously expressed, a recent study demonstrated that increased NFκB activity in astrocytes decreases the transcription of catechol-*o*-methyltransferase (COMT), which is an enzyme that inactivates catecholamines that cause pain.[41] Reduced COMT activity is related to enhanced pain sensitivity in patients with chronic pain conditions.[41,42]

The role of NFκB in the pathogenesis of CRPS is not well understood. One study has investigated changes in NFκB in a CRPS animal model.[24] NFκB was measured in the extracts of muscle and spinal cord tissue, and it was shown that NFκB is elevated in muscle and spinal cord of CPIP rats when compared to sham-treated rats. Moreover, systemic administration of an NFκB inhibitor reduced allodynia in a dose-dependent manner. These findings suggest that NFκB in muscle and spinal cord plays a role in the pathogenesis of CRPS. The abovementioned study was focused on the roles of NFκB in the regions below the brain. Hence, our study is the first to characterize changes in NFκB in the cerebrum in a CRPS animal model. The abovementioned study showed that the level of spinal NFκB increased just 2 hours after the CPIP procedure. Therefore, we can postulate that cerebral NFκB also increases within a few hours after CPIP model generation, which may be related to the mechanical allodynia in the early phase after CPIP procedure.

We measured the MTs during the post-reperfusion period at 1 hour, 4 hours, 24 hours, 48 hours, 7 days, and 21 days. The time points for data collection were determined based on the previous studies. In the first study on CPIP model generation, Coderre et al described that mechanical allodynia appeared within 8 hours and continued till at least 4 weeks after the procedure.[27] Furthermore, 14 days after CPIP model generation are considered to be a "chronic" phase.[43] Another study examined mechanical allodynia on day 1, day 2, day 7, and day 21 after the procedure.[6] Hence, based on these studies, day 21 was considered to be sufficient for confirming the generation of the CPIP model, and we classified the animals into CPIP-positive and CPIP-negative groups on day 21 after the application of the O-ring.

In this study, a CPIP model was used because the signs and symptoms of CRPS can be observed without direct nerve injury or fracture.[27] It is thus a widely used animal model for CRPS.[6,27,44,45] Considering that the symptoms and signs of CRPS type 1 may develop after minor tissue injury without direct nerve injury or fracture, it is appropriate to use this model in studies of CRPS in animals.

Our study has some limitations. First, the cerebral region in which increased NFκB expression occurs is unclear. The authors analyzed the right cerebrums of the rats. It was not easy to determine the exact region of the rat cerebral cortex due to its size and the lack of information on the functional map of the rat cerebral cortex. Complementary studies including either immunohistochemistry or quantitative PCR (qPCR) from the subregion of interest are required. Functional imaging studies can be used to characterize different brain areas in CRPS. The activation patterns are different in patients with CRPS when compared to healthy subjects in the sensorimotor cortex, inferior frontal gyrus, and secondary somatosensory cortex.[46,47] Another limitation of our study is that the exact role of the increased NFκB in the cerebrum is unclear. The elevated NFκB may not be pain specific. However, considering that increased NFκB is associated with long-term potentiation and synaptic plasticity in the central nervous system, the authors can postulate that increased NFκB can result in the initiation and progress of CRPS. Finally, we measured the MT and NFκB levels without using NFκB antagonists. This is because we only wanted to confirm the results derived from the bioinformatics and text-mining study,[7] which indicated that NFκB may be a central mediator in CRPS. The finding that NFκB levels are increased in the cerebral cortex in CPIP animals can be the first step toward further studies evaluating the role of NFκB in CRPS pathogenesis. Further studies using an NFκB inhibitor are required to elucidate the signaling pathway involved in CRPS.

## Conclusion

Our results suggest that peripheral injury can increase cerebral NFκB expression and that NFκB might play a role in the

pathogenesis of pain in the CPIP animal model and in human CRPS. Further studies on the molecular pathways involving NFκB in CRPS are required to elucidate the pathogenesis of CRPS and to determine a novel target for the treatment of CRPS.

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## Disclosure

The authors report no conflicts of interest in this work.

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