Phosphorylated CaMKII levels increase in rat central nervous system after large-dose intravenous remifentanil

BCEF 1 Qiang Wang*
BCEF 1 Xin Zhao*
ADFG 1 Shuren Li
BC 2 Song Han
BC 2 Zhifeng Peng
ADEFG 2 Junfa Li

* Q. Wang and X. Zhao contributed equally to this work

Corresponding Author: Junfa Li, e-mail: jufal@ccmu.edu.cn and Shuren Li, e-mail: csa8@china.com

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Background: Postoperative remifentanil-induced pain sensitization is common, but its molecular mechanism remains unclear. Calcium/calmodulin-dependent protein kinase II (CaMKII) has been shown to have a critical role in morphine-induced hyperalgesia. This study was designed to determine how CaMKII phosphorylation and protein expression levels change in the central nervous system of rats with remifentanil-induced hyperalgesia.

Material/Methods: Male Sprague-Dawley® rats were exposed to large-dose (bolus of 6.0 µg/kg and 2.5 µg/kg/min for 2 hours) intravenous remifentanil to induce post-transfusion hyperalgesia. Levels of phosphorylated CaMKII (P-CaMKII) and total protein of CaMKII (T-CaMKII) were determined at different post-transfusion times by Western blot and immunostaining and were compared with controls.

Results: P-CaMKII increased significantly (P<0.05) at 0, 0.5, and 2 hours. However, P-CaMKII at 5 to 24 hours and T-CaMKII at 0 to 24 hours post-transfusion did not change significantly in rats’ spinal dorsal horn, hippocampus, or primary somatosensory (S1) cortex (n=6 per group). Similarly, immunostaining showed stronger P-CaMKII immunoreactants (P<0.05) and more P-CaMKII- positive cells (P<0.05) in the spinal dorsal horn, CA1 region of the hippocampus, and S1 cortex of rats 0.5 hours post-transfusion compared with the control group treated with 0.9% sodium chloride (n=3 per group).

Conclusions: These results suggest that a temporary rise in the P-CaMKII level in the central nervous system may correlate with remifentanil-induced pain sensitization in the postoperative period.

Key words: remifentanil • hyperalgesia • central nervous system • calcium/calmodulin-dependent protein kinase II (CaMKII)

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Background

Remifentanil, an ultra-short-acting opioid, is widely used in clinical practice. However, severe postoperative pain, or opioid-induced hyperalgesia, often occurs when remifentanil is discontinued, most likely as the result of nociceptive sensitization [1,2]. Research has elucidated the factors responsible for the paradoxical effects of opioids, but neurobiological evidence of remifentanil-induced hyperalgesia remains scarce.

The N-methyl-D-aspartate (NMDA) receptor is known to be critical in mediating calcium (Ca$^{2+}$) signaling and may contribute to the development and maintenance of hyperalgesia induced by discontinuation of remifentanil or other opioids [3–5]. Direct phosphorylation of the NMDA receptor is crucial in regulating the channel function and localization of NMDA receptors at synapses [6]. Some serine/threonine phosphorylation sites that are substrates for protein kinase C (PKC) or Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) have been identified in NMDA receptors [7]. A positive feedback loop shows that the activation of NMDA receptors leads to increased intracellular Ca$^{2+}$ levels and the subsequent activation of PKC and CaMKII. PKC and CaMKII can in turn activate NMDA receptors by phosphorylation, which is the proposed mechanism for neuropathic pain [8] and opioid tolerance [9]. In previous studies, we demonstrated that conventional PKC (cPKC) γmembrane translocation is involved in remifentanil-induced hyperalgesia [10]. Moreover, PKC is often consistent with CaMKII in regulating neural plasticity [11–13]. Therefore, in this study, we explored how CaMKII levels change with remifentanil exposure.

CaMKII, a multifunctional serine/threonine protein kinase diffused throughout the central nervous system, is encoded by 4 genes; its α subunit has an important role in regulating neuronal plasticity [14,15]. Elevated intracellular Ca$^{2+}$ and calmodulin levels are essential in the activation of CaMKII. The activated CaMKII begins to phosphorylate itself and protein subunits such as neuronal membrane receptors and intracellular transcription factors [16,17]. Inhibition of CaMKII activity has been shown to reverse morphine tolerance and dependence in a dose-dependent manner [18]. Spinal CaMKII activity increased in subcutaneous injection of a morphine-induced hyperalgesia mouse model, whereas mechanical allodynia and thermal hyperalgesia were not detected in CaMKIIα T286A mutant mice [19].

Upregulation of CaMKII has been implicated in neuropathic and inflammatory pain. CaMKII inhibitors reversed thermal hyperalgesia and mechanical allodynia in these experimental models in a dose-dependent manner [20–22]. In addition, patients have reported less pain after discontinuation of remifentanil when the commonly used anesthetics propofol or ketamine were added to the treatment protocol [23,24]. Both propofol and ketamine have been demonstrated to inhibit CaMKII phosphorylation in a dose-dependent manner [25]; therefore, determination of how CaMKII activity changes with remifentanil administration will help identify the therapeutic target for opioid-induced hyperalgesia.

The spinal dorsal horn, rather than the hippocampus or cortex, is typically the focus of studies of opioid-induced hyperalgesia. However, intravenous administration of remifentanil is a systemic event, and some research supports the involvement of the hippocampus and primary somatosensory (S1) cortex in hypersensitivity and hyperalgesia. For example, fentanyl exposure increases the susceptibility of CA1 pyramidal neurons to presynaptic stimulation [26]. Altered sensory processing related to hyperalgesia is reflected in laser C-fiber-evoked potentials in the forelimb and hind limb S1 cortex [27]. Induction of spinal long-term potentiation (LTP), which may contribute to hypersensitivity and hyperalgesia, first causes an acute metabolic response in the S1 cortex rather than in other regions of the brain [28]. Expression of the CaMKII gene in the rat hippocampus and frontal cortex has been found to be upregulated after repeated morphine administration [29]. Therefore, the objective of this study was to investigate how levels of CaMKII phosphorylation at threonine 286 (P-CaMKII) and total protein (T-CaMKII) change in the spinal dorsal horn, hippocampus, and S1 cortex of rats with remifentanil-induced hyperalgesia.

Material and Methods

Experiments were carried out on 8- to 10-week-old male Sprague-Dawley® rats weighing 240 to 260 g. The rats were initially housed in a controlled room at 21°C to 24°C with a 12:12 hour light-dark cycle. Rats had ad libitum access to food and water. The animal protocol was approved by the Institutional Animal Care and Use Committee of Capital Medical University and is consistent with the ethical guidelines of the International Association for the Study of Pain for pain research in conscious animals.

The following materials were obtained from the indicated sources: phosphatase inhibitors (okadaic acid, sodium pyrophosphate, and potassium fluoride); proteinase inhibitors (leupeptin, aprotinin, pepstatin A, and chymostatin); mouse monoclonal antibodies against β-actin; ethylene diaminetetraacetic acid (EDTA); ethylene glycol tetraacetic acid (EGTA); sodium dodecyl sulfate (SDS); dithiothreitol (DTT); Nonidet™ P-40 (Sigma-Aldrich, St. Louis, MO, USA); horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Bio-Rad, Hercules, CA, USA); and remifentanil (Yichang Humanwell Pharmaceutical Co LTD, PR China).

Drug administration

Rats were assigned to a treatment group that received intravenous remifentanil, or to a control group that received...
intravenous 0.9% sodium chloride via a tail vein. Intravenous lines were established through a tail vein via a 24-G trocar (Suzhou Becton Dickinson Medical Devices Co. Ltd., PR China). After successful cannulation, infusion pumps (Graseby 3500 Anesthesia Syringe Pump, Graseby Medical Ltd., UK) were connected for transfusion. The treatment protocol was performed as follows: rats in the control group received 0.9% sodium chloride with a bolus of 0.3 mL/kg at 0.125 mL/kg/min; rats in the treatment group received remifentanil with a bolus of 6.0 µg/kg at 2.5 µg/kg/min (a large dose with 20 µg/mL concentration). The transfusion lasted 2 hours, and spontaneous breaths and eyelash reflexes were monitored to ensure the rats' well being.

**Mechanical sensitivity test**

Before the transfusion, rats in the control group (n=10) and treatment group (n=10) were separately placed in a Plexiglas® container (25×25×25 cm) with a wire mesh bottom and allowed to acclimate for 30 minutes. The basal value of the paw withdrawal threshold (PWT) of each rat was measured by applying a probe to the plantar surface of the hind paw with an electronic von Frey aesthesiometer until a withdrawal response occurred. A cut-off value for the PWT was set at 120 g to prevent mechanical injury. After the transfusion, the PWT was measured by the same method at 0, 0.5, 2, 5, and 24 hours post-transfusion. The PWT of each rat was tested 3 times at different post-transfusion time points. The mean value of the 3 measurements was used.

**Sample preparation and Western blot analysis**

After the transfusion, rats in the control (n=6) and treatment groups were sacrificed 0, 0.5, 2, 5, and 24 hours (n=6 per group) post-transfusion. The rats were anesthetized with 10% chloral hydrate, and a cannula was inserted into the left ventricle of each rat 0.5 hours post-transfusion [10]. After cannulation, 100 mL 0.9% NaCl and 250 mL 10% neutral buffered formalin 250 mL were perfused. Spinal cords and brains were quickly removed and placed into ice-cold artificial cerebrospinal fluid (NaCl 125 mM, KCl 2.5 mM, CaCl 2.0 mM, NaHCO 26 mM, NaHPO 1.25 mM, MgCl 1.0 mM, glucose 5 mM, pH 7.4) with 95% O 2 and 5% CO 2. The dorsal horn area of the lumbosacral enlargement, hippocampus, and S1 cortex were identified and collected as required, and then they were frozen in liquid nitrogen and kept at −80°C for analysis.

As previously reported [30], the frozen samples were rapidly thawed, homogenized at 4°C in Buffer C (50 mM Tris-Cl, pH 7.5) containing 2 mM DTT, 2 mM EDTA, 2 mM EGTA, 50 mM 4-[2-aminoethyl]-benzenesulfonylfluoride hydrochloride; 5 mg/mL each of leupeptin, aprotinin, pepstatin A, and chymostatin; 50 mM KF, 50 mM okadaic acid, 5 mM sodium pyrophosphate, and 2% SDS, and sonicated to dissolve the tissue completely. Protein concentration was measured with a BCA Protein Assay Kit (Thermo Scientific, Pittsburgh, PA, USA).

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis were performed as in a previous study [25]. Briefly, 35 µg of total protein in each sample was loaded in the corresponding lane for a 10% SDS-PAGE gel. After electrophoresis and transfer of proteins onto polyvinylidene difluoride membrane (PVDF, GE Healthcare, USA) at 4°C, the PVDF membrane was blocked with 10% nonfat milk in TTBS (20 mM Tris-Cl, pH 7.5, containing 0.15 M NaCl and 0.05% Tween 20) for 1 hour. The blocked membrane was incubated with primary rabbit polyclonal antibody against P-CaMKII (T286, 1:1000) for 3 hours or for 1 hour in T-CaMKII (1:1000, Santa Cruz Biotechnology, Inc., USA). To ensure uniform loading of protein, the same PVDF membrane was reprobed with primary mouse monoclonal antibody against β-actin (Sigma-Aldrich, USA) at a 1:2000 dilution for 1 hour. The horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (GE Healthcare, USA) were used as second antibodies at a 1:4000 dilution and incubated for 1 hour. An enhanced chemiluminescence kit (PerkinElmer Life Science, USA) was used to identify the signals on radiographic film. The sequence to detect the target protein is P-CaMKII, T-CaMKII, β-actin. Before starting each round of detection on the same PVDF membrane, stripping buffer containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) at 55°C was applied and the membrane incubated until no signals appeared in the radiographic film, indicating that the previously bonded antibodies had been stripped from the membrane.

**Immunostaining**

Post-fixed spinal cords and brains were embedded in paraffin, and serial 4-µm sections were prepared with a microscope. Immunohistochemistry was performed using a PV-6000 kit (Zymed Co., USA). To detect P-CaMKII expressions in the spinal cord, hippocampus, and cortex, the sections were de-waxed and hydrated in xylene and graded alcohol solution.

Sections underwent an antigen-retrieval treatment in a pressure cooker for 5 minutes and were treated with 0.3% hydrogen peroxide for 15 minutes at room temperature to inhibit endogenous peroxidase activity. Sections were incubated with primary rabbit polyclonal antibody against P-CaMKII (T286, Santa Cruz Biotechnology, Inc., USA) for 24 hours in a 1:100 dilution at 4°C. Incubated sections were washed with phosphate buffer solution (PBS) 3 times. An immunoreaction was triggered with a PV-6000 kit and visualized with peroxidase-3,3’-diaminobenzidine. Finally, sections were counterstained with haematoxylin and eosin.
and mounted. Identical spinal cord and brain samples from controls were set with PBS, rather than the primary antibodies, and incubated. The images were captured with a Leica microscope imaging system (Leica, Wetzlar, Germany). Three selected fields in 3 serial sections were analyzed under a light microscope (10×40 magnification).

Statistical analysis

The within-group difference of rats’ PWT before and after the transfusion was analyzed for mechanical sensitivity. Quantitative analysis for Western blot results was performed after scanning the radiographic film with Quantitative One software (Gel Doc-2000, Bio-Rad, USA). To determine the T-CaMKII and P-CaMKII levels, the relative optical density (ROD) of T-CaMKII or P-CaMKII bands were normalized against that of β-actin or T-CaMKII, respectively, and expressed as percentage of the control group (100%). For immunostaining, the P-CaMKII expression was determined as the mean integrated optical density (IOD, mean IOD=IOD/area) of captured images using Image-Pro Plus 6.0 software (Media Cybernetics, Inc, USA). The presented values are expressed as mean ±SE. Statistical analysis was conducted by one-way analysis of variance (ANOVA) followed by pairwise multiple comparison procedures using the Bonferroni test. The significance level was set at P<0.05.

Results

PWT decreased significantly (P<0.05) at 0 hours (56.01±0.89 g), 0.5 hours (37.61±1.13 g), and 2 hours (33.73±1.23 g) after transfusion of remifentanil, and it remained stable in control rats (n=10 per group) (Figure 1).

Changes in P-CaMKII and T-CaMKII levels in the spinal dorsal horn, hippocampus, and S1 cortex

P-CaMKII and T-CaMKII were detected at 52.0 KD with Western blot (Figure 2A). Quantitative analysis (Figure 2B) showed that P-CaMKII increased significantly (P<0.05, n=6 per group) at 0 hours (192±22), 0.5 hours (196±17), and 2 hours (165±18) after transfusion with remifentanil in the spinal dorsal horn, compared with that of the control group (100%).

Similarly, the P-CaMKII levels increased significantly (P<0.05, n=6 per group) in the hippocampus (Figure 3A and 3B) and S1 cortex (Figure 4A and 4B) of rats at 0 hours (hippocampus: 232±22; S1 cortex: 231±37), 0.5 hours (hippocampus: 225±11; S1 cortex: 239±68), and 2 hours (hippocampus: 281±26; S1 cortex: 284±23), while it remained stable in control rats (n=10 per group).
cortex: 232±62) after transfusion with remifentanil. However, no significant changes in levels of P-CaMKII at 5 to 24 hours or T-CaMKII at 0 to 24 hours after transfusion with remifentanil were observed in the spinal dorsal horn, hippocampus, or S1 cortex of rats (Figures 2–4).

**Discussion**

Some investigators have reported that Remifentanil-induced hyperalgesia is easier to induce with high doses than with low doses [31–33]. Continuous intravenous transfusion of remifentanil at 0.4 µg/kg/min, with or without a bolus of 1.0 µg/kg, is regarded as a large dose in clinical practice and has been demonstrated to increase postoperative pain sensitivity [34–36]. The equivalent dose used in rats for Western blot and immunohistochemistry analysis in this study was converted according to the experimental formula in FDA (2005) Guidance for

**Figure 3.** Changes in P-CaMKII and T-CaMKII levels in the hippocampus at 0 to 24 hours post-transfusion of large-dose intravenous remifentanil. (A) Typical Western blot showed changes in P-CaMKII and T-CaMKII in the hippocampus of rats at 0 to 24 hours post-transfusion with remifentanil; (B) Quantitative analysis demonstrated a significant increase of P-CaMKII at 0, 0.5, and 2 hours, but no significant changes in P-CaMKII at 5 to 24 hours or T-CaMKII at 0 to 24 hours post-transfusion with remifentanil were observed in the hippocampus. * P<0.05 vs. control (100%), n=6 per group.

**Figure 4.** Changes in P-CaMKII and T-CaMKII levels in the S1 cortex at 0 to 24 hours post-transfusion of large-dose intravenous remifentanil. (A) Typical results of Western blot showed changes in P-CaMKII and T-CaMKII levels in the S1 cortex of rats at 0 to 24 hours post-transfusion with remifentanil; (B) Quantitative analysis demonstrated a significant increase in P-CaMKII at 0, 0.5, and 2 hours, but no significant changes in levels of P-CaMKII at 5 to 24 hours or T-CaMKII at 0 to 24 hours post-transfusion with remifentanil were observed in the S1 cortex. * P<0.05 vs. control (100%), n=6 per group.

**Distribution of P-CaMKII-positive cells in the spinal cord, hippocampus and S1 cortex**

To determine the distribution of P-CaMKII in the spinal cord, hippocampus, and S1 cortex of rats after intravenous administration of remifentanil, we performed immunostaining in 3 rats each in the treatment group and control group at 0.5 hours post-transfusion. Similar to the results of Western blot analysis, stronger P-CaMKII immunoreactants (mean IOD, P<0.01) and more P-CaMKII-positive cells (P<0.05) were observed in spinal dorsal horn (Figure 5B1 and 5B2), the CA1 region of the hippocampus (Figure 5D1 and 5D2), and S1 cortex (Figure 5F1 and 5F2) at 0.5 hours after transfusion with remifentanil (Figure 5G and 5H). Significantly increased P-CaMKII levels were found mainly in the cytoplasm.
Industry Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers (rat dose = human equivalent dose × 6.2). The finding that half a clinical large dose (bolus of 6.0 µg/kg and a transfusion at 1.2 µg/kg/min) had triggered hyperalgesia [10], taken with the results of the mechanical sensitivity test in this study, increased levels of P-CaMKII after a large-dose intravenous transfusion with remifentanil may reflect a correlation between CaMKII activity and hyperalgesia.

A rat model of incision pain is often used in the investigation of postoperative pain in humans [37]. Because remifentanil-induced hyperalgesia occurred primarily in postoperative patients, the plantar incision pain model is commonly used to reproduce hyperalgesia in clinical practice [10;38]. However, a surgical procedure itself could also induce thermal hyperalgesia and mechanical allodynia [39,40], and incision-evoked hyperalgesia was found to be closely related with spinal CaMKII activation [41]. Based on this evidence, the non-incision model further reduces confounding factors and better reflects the relationship between remifentanil exposure and changes in CaMKII levels in the central nervous system. Therefore, a non-incision model was chosen for this study.

Through the detection of changes in P-CaMKII and T-CaMKII levels in the spinal cord, hippocampus, and S1 cortex through Western blot and immunohistochemical analysis, we have formed 3 conclusions. First, short-term exposure to remifentanil at the dose that tends to induce postoperative hyperalgesia in clinical practice could enhance CaMKII activity by increasing P-CaMKII rather than T-CaMKII levels. Second, the spinal dorsal horn is critical but may not be the only important site involved in remifentanil-induced hyperalgesia; supraspinal structures such as the hippocampus and S1 cortex may
also be responsible. Third, the effect of remifentanil on CaMKII activity must be temporary (less than 5 hours) to maintain the P-CaMKII at a higher level.

A series of studies have demonstrated the importance of supraspinal structures in hyperalgesia [42–46]. As an important regulator, levels of P-CaMKII increased in both the spinal dorsal horn and the hippocampus during the nociceptive processes of inflammatory pain [47,48]. In a rodent model of morphine antinociceptive tolerance and physical dependence, tissues from the frontal cortex and lumbar spinal sections show increased activity, but not expression, of CaMKII [49]. A clinical observation has also revealed that the visual analogue scale at rest was significantly increased from only 0 to 2 hours postoperatively in the remifentanil group [35]. In addition, our finding of increased levels of P-CaMKII but not T-CaMKII in the spinal dorsal horn, hippocampus, and S1 cortex 2 hours after transfusion of remifentanil is consistent with findings of a report of morphine antinociceptive tolerance [50].

Among all possible causes of remifentanil-induced hyperalgesia, the NMDA receptor is most often mentioned [3–5,51]. A large-dose transfusion of remifentanil has been shown to enhance the function of NMDA receptors via activation of the δ-opioid receptor, resulting in remifentanil-induced hyperalgesia [4]. Although no findings have elucidated the mechanism by which CaMKII interacts with the NMDA receptor in remifentanil-induced hyperalgesia, some reports support the interaction between CaMKII and the NMDA receptor in neuropathic pain. For example, the disruption of CaMKII docking on the NMDA receptor did not induce neuropathic behavioral reflex sensitization [52]; lack of phosphorylation of NR2B subunits of the NMDA receptor at Tyr1472 in the dorsal horn impaired CaMKII activation and attenuate neuropathic pain [53]. A non-selective NMDA receptor antagonist significantly decreased CaMKII levels in a chronic constriction injury model [20]. In addition, it was reported that neuroprotection against ischemic injury could be induced by diminishing the translocation of CaMKII from cytoplasm to cell membrane [54]. Whether inhibition of the membrane translocation of P-CaMKII is a therapeutic target for opioid-induced hyperalgesia should be the subject of future research.

As indicated in the results that increased P-CaMKII restored to the baseline 5 hours after surgery, an optimal drug to inhibit the increased P-CaMKII should be short-lasting, otherwise the state of “decreased P-CaMKII” may occur. As previously described, propofol, a short-lasting anesthetic, has been demonstrated to inhibit CaMKII phosphorylation in an anesthetic depth-dependent way and to provide better postoperative analgesia when it was added to the treatment protocol in patients after remifentanil-based anesthesia. Therefore, it may be an optimal drug in remifentanil-induced hyperalgesia.

Conclusions

This study demonstrated that large-dose intravenous transfusion of remifentanil can temporarily increase P-CaMKII levels in the central nervous system, which may correlate with postoperative pain sensitization. These results also suggest that drugs chosen for treatment of remifentanil-induced hyperalgesia should be short-acting rather than long-acting.
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