Spinal Protein Kinase ζ Regulates α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor Trafficking and Dendritic Spine Plasticity via Kalirin-7 in the Pathogenesis of Remifentanil-induced Postincisional Hyperalgesia in Rats

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ABSTRACT

Background: Intraoperative remifentanil anesthesia exaggerates postoperative pain sensitivity. Recent studies recapitulate the significance of protein kinase ζ in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor–mediated pathologic pain. Kalirin-7, a Rho guanine nucleotide exchange factor, coordinates AMPA receptor trafficking and dendritic spine plasticity. This study examines whether protein kinase ζ and Kalirin-7 contribute to remifentanil-induced postincisional hyperalgesia via AMPA receptor.

Methods: Plantar incision was performed 10 min after the start of remifentanil infusion (1 μg · kg⁻¹ · min⁻¹ for 60 min). Paw withdrawal threshold (primary outcome), spinal protein kinase ζ activity, Kalirin-7 expression, AMPA receptor trafficking, and spine morphology were assessed. Protein kinase ζ inhibitor and Kalirin-7 knockdown by short hairpin RNA elucidated the mechanism and prevention of hyperalgesia. Whole-cell patch-clamp recording analyzed the role of protein kinase ζ in spinal AMPA receptor–induced current.

Results: Remifentanil reduced postincisional paw withdrawal threshold (mean ± SD, control vs. hyperalgesia, 18.9 ± 1.6 vs. 5.3 ± 1.2 g, n = 7) at postoperative 48 h, which was accompanied by an increase in spinal protein kinase ζ phosphorylation (97.8 ± 25.1 vs. 181.5 ± 18.3%, n = 4), Kalirin-7 production (101.9 ± 29.1 vs. 371.2 ± 59.1%, n = 4), and number of spines/10 μm (2.0 ± 0.3 vs. 13.0 ± 1.6, n = 4). Protein kinase ζ inhibitor reduced remifentanil-induced hyperalgesia, Kalirin-7 expression, and GluA1 trafficking. Incubation with protein kinase ζ inhibitor reversed remifentanil-enhanced AMPA receptor-induced current in dorsal horn neurons. Kalirin-7 deficiency impaired remifentanil-caused hyperalgesia, postsynaptic GluA1 insertion, and spine plasticity. Selective GluA2-lacking AMPA receptor antagonist prevented hyperalgesia in a dose-dependent manner.

Conclusions: Spinal protein kinase ζ regulation of GluA1-containing AMPA receptor trafficking and spine morphology via Kalirin-7 overexpression is a fundamental pathogenesis of remifentanil-induced hyperalgesia in rats. (Anesthesiology 2018; 129:173-86)

REMIFENTANIL, as a potent short-acting μ-opioid, is a balanced anesthesia component in clinical setting.1,2 The intraoperative exposure to remifentanil elicits opioid-induced hyperalgesia in animals.3–5 Although the clinical importance and effective prevention of opioid-induced hyperalgesia is poorly understood,6,7 the enhanced postoperative nociceptive sensitivity is considered a major potential factor for the development of chronic pain and impairing chronic pain treatment with opioids in patients.8–11 Therefore, additional studies on the specific mechanism underlying opioid-induced hyperalgesia are essential, which might contribute to improving the clinical consequences.

What We Already Know about This Topic
- Intraoperative exposure to short acting opioids may increase postoperative pain levels
- In animal models, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors contribute to opioid-enhanced nociceptive sensitivity

What This Manuscript Tells Us That Is New
- The inhibition of protein kinase ζ prevents remifentanil-enhanced postoperative hyperalgesia in a rat incisional model
- The intraoperative effects of remifentanil in this rat model involve changes in dendritic spine morphology and function in the dorsal horn of the spinal cord
Protein Kinase Mζ, N-terminally truncated and constitutively active form of the atypical protein kinase C, can sustain the synaptic potentiation via increasing and maintaining the synaptic AMPA receptor.12,17–19 Protein kinase Mζ is identified as a critical modulator in several hypernociceptive models.14,20–22 Li et al.23 described the impairment of neuropathic pain by inhibiting protein kinase Mζ via a reduced level of postsynaptic GluA1 in the anterior cingulated cortex. Recently, we found that spinal protein kinase Mζ activation mediated by N-methyl-D-aspartate (NMDA) receptor subunit 2B (NR2B)–containing NMDA receptor is required for remifentanil-induced postinfusion hyperalgesia;24 however, only little is known about the downstream target of protein kinase Mζ in this pathway. Kalirin-7, an isoform of Rho guanine nucleotide exchange factors, is recognized for its pivotal influence on the structural and functional plasticity of excitatory synapses.25 Kalirin-7–dependent dendritic spine formation and enlargement is essential for the regulation of the synaptic strength.26,27 Kalirin-7 also binds directly with GluA1 to facilitate AMPA receptor expression at the synaptic sites.28–30 Moreover, protein kinase Mζ is involved in the modification of spine structure and function, which plays a role in underlying synaptic and behavioral plasticity.31 However, the overall link among spinal protein kinase Mζ activity, Kalirin-7 overexpression, and AMPA receptor trafficking in opioid-induced hyperalgesia is unclear.

In this present study, we investigated the effect of protein kinase Mζ on AMPA receptor trafficking and postoperative hyperalgesia induced by remifentanil anesthesia. Specifically, we examined the expression and phosphorylation of protein kinase Mζ in the dorsal horn, and we evaluated the level of spinal Kalirin-7 and dendritic spine morphology. Furthermore, the pharmacologic inhibition of protein kinase Mζ and knockdown of Kalirin-7 with intrathecal short hairpin RNA verified the mechanism and prevention of hyperalgesia. Herein, we revealed the involvement of Kalirin-7 in nociceptive transduction and transmission by mediating the protein kinase Mζ-dependent AMPA receptor surface expression.

Materials and Methods

Animals

All protocols were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University (Tianjin, China). The procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Adult (8- to 10-week-old) and young (5- to 7-week-old) male Sprague–Dawley rats were used for the experimental studies. The animals were purchased from the Laboratory Animal Center of the Military Medical Science Academy of the Chinese People’s Liberation Army and maintained (fewer than five rats/cage) under a 12-h light/dark cycle with food and water ad libitum. Acclimatization was allowed for 7 days before initiating any experiment. The investigators were blinded to the randomized grouping of the animals and treatment conditions for all the experiments.

Reagents

Remifentanil hydrochloride (batch number 6170705; RenFu Pharmaceutical Co., China) was solubilized in saline (1 mg of remifentanil in 100 ml of saline) and intravenously administered 1 µg · kg⁻¹ · min⁻¹ for 60 min under sevoflurane anesthesia (induction, 3.0%; surgery, 1.5%; Maruishi Pharmaceutical Co., Ltd., Japan) using a nose mask. The dose of remifentanil was selected based on previous reports.3,4,32 Intravenous saline was infused 0.1 ml · kg⁻¹ · min⁻¹ for 60 min as control exposure. Protein kinase Mζ inhibitor ζ-pseudosubstrate inhibitory peptide, scrambled ζ-pseudosubstrate inhibitory peptide from Tocris (USA), and a selective GluA2-lacking AMPA receptor antagonist 1-naphthylacetyl spermine trihydrochloride (NASPM; Sigma–Aldrich, USA) were delivered intrathecally before remifentanil infusion. The intrathecal application with reagents (10 µl) was performed through the skin was sutured with 4-0 silk thread and then covered.

Plantar Incision

The incisional postoperative pain model was established according to the previously described procedures.3,4,32 Briefly, animals were anesthetized with sevoflurane (induction, 3.0%; surgery, 1.5%) for 60 min using a nose mask, and the tail arterial blood pressure was also detected using a noninvasive blood pressure monitor (CODA monitor, Kent Scientific Corp., USA). A 1-cm longitudinal incision was made through the skin and fascia of the plantar, beginning at 0.5 cm from the edge of the heel and extending toward the toes of the left hind paw. The plantaris muscle was elevated using forceps, the muscle origin was maintained, and the insertion was integral. After compression hemostasis, the skin was sutured with 4-0 silk thread and then covered.

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is an ionotropic glutamatergic receptor composed of four independent subunits (GluA1–4); it is implicated in excitatory synaptic plasticity through alterations of their number, composition, and translocation to synapses in the central nervous system.12 Several studies have recapitulated that both peripheral inflammation and nerve injury triggered GluA1-containing AMPA receptor membrane trafficking at synapses of spinal dorsal horn neurons, which may elicit acute and persistent inflammatory as well as neuropathic pain.13–15 Also, our previous study highlights the remifentanil-induced trafficking of extrasynaptic GluA1-containing AMPA receptor in the spinal cord during the development of opioid-induced hyperalgesia;16 however, the specific molecular modulation of AMPA receptor trafficking is not yet reported.

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with erythromycin ointment for the prevention of infection. The incision was made 10 min after the start of intravenous remifentanil or saline infusion. The rats with sham operations underwent identical anesthesia exposure without an incision.

Kalirin-7 Knockdown
A previously characterized short hairpin RNA, which targets the Kalirin-7 sequence GCAGTACAATCCTGGCCCATGT (beginning at position 1,229), was cloned into a GV248 lentiviral vector. The intrathecal injection using the virus construct lentivector–Kalirin-7–short hairpin (10 µl; 3 × 10^6 transducing units/ml) was applied to knockdown the expression of endogenous Kalirin-7. Moreover, lentiviral vector-containing the negative control sequence TTCTC- CGAAGCTGTAGCGT was administrated intrathecally (10 µl) as control treatment. The lentiviral vector–Kalirin-7–short hairpin RNA and lentiviral vector–negative control were designed and supplied by GeneChem (China). After 1-week recovery, those rats that exhibited any deficiency in the locomotor function were discarded. To verify the efficiency of blockage in Kalirin-7 expression, the dorsal horn of spinal cord segment L₄ to L₆ was obtained for Western blot after 2 and 3 weeks, respectively.

Behavioral Testing
To test the mechanical sensitivity, rats were confined in elevated cages with wire mesh bottoms and allowed acclimatization for 1 h. Electronic Von Frey filaments (BSEVF3, Harvard Apparatus Co., USA) were applied vertically to stimulate the plantar bordering the incision of the left hind paw; the experiments were repeated three times at 3-min intervals at each time point as described previously. The paw withdrawal threshold was determined as the mean pressure (g) of three trials: when rats shook, withdrew, or licked their paws. A maximal cutoff threshold of 50 g was set to avert tissue damage.

The hot plate (YLS-6B, Zhenghua Biologic Instrument Equipment Co., Ltd., China) evaluated the thermal sensitivity. The time the rat spent on the hot plate at 52°C before showing a clear paw withdrawal, shaking, or licking was recorded and measured at each time point. The paw-withdrawal latency was calculated as the average time (s) of three repeated experiments. A cutoff time of 30 s was used to avoid damage to the hind paws.

The locomotor function was observed by assessing the placing, grasping, and righting reflex as previously described. To assess the placing reflex, the hind limbs of the rat were held slightly lower than the forelimbs, the dorsal surfaces of the hind paws were brought into contact with the edge of a table, and whether the hind paws were placed on the table surface reflexively was assessed. To assess the grasping reflex, the rat was placed on a wire grid and whether the hind paws grasped the wire on contact was recorded. To assess the righting reflex, the back of the rat was placed on a flat surface, and whether it immediately reverted to the normal upright position was evaluated. Every test was repeatedly conducted for six trials, and the counts of each normal reflex were considered as scores for the tests. All behavioral data were collected and recorded by the same investigator to eliminate observational biases.

Western Blot
After the proper survival times, the animals were euthanized under deep sevoflurane (3%) anesthesia. The L₄–L₆ spinal cord segments were removed rapidly and snap-frozen in liquid nitrogen. The left dorsal horn was mechanically homogenized in ice-cold radioimmune precipitation assay buffer containing phenylmethanesulfonyl fluoride (Abcam, United Kingdom). The lysate was centrifuged, and the supernatant was collected as the total protein. A membrane compartment protein extraction kit (BioChain Institute, Inc., USA) was used to extract the membrane fraction of the dorsal horn. The protein content was determined using the bicinchoninic acid assay method. The loading and blotting of an equivalent amount of membrane and total proteins were verified using a membrane with monoclonal mouse anti–N-cadherin (135 kd; 1:2,000; Sigma–Aldrich, USA) and anti-β-actin antibody (42 kd; 1:5,000; Sigma–Aldrich, USA), respectively. The samples were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane, and probed with polyclonal rabbit antibodies against rat protein kinase Cζ, GluA1, GluA2 (55, 100, and 102 kd, respectively; all 1:2,000; Abcam), phosphorylated protein kinase Cζ at Thr410 (1:2,000; Cell Signaling Technology, USA), and goat polyclonal anti-Kalirin (around 200 kd; 1:1,000; Abcam), followed by incubation with horseradish peroxidase–conjugated secondary antibodies (1:2,000; Jackson ImmunoResearch, USA). The membrane-bound secondary antibodies were visualized with enhanced chemiluminescence (Thermo Scientific, USA) and quantified using Gene Tools Match software (Syngene, United Kingdom). The results were quantified through two steps. First, protein amount was calculated as the percentage of endogenous control (β-actin or N-cadherin). Second, the protein levels obtained from the treatment were expressed as percentages compared to control condition.

Golgi Staining
As described previously, the freshly dissected spinal dorsal horns were immersed in 20 ml of a Golgi–Cox solution for 2 weeks at room temperature. Then the dorsal horns were transferred into 30% sucrose for a minimum of 2 days to enhance the pliability. Sections (100 µm) were placed on 2% gelatinized microscope slides, rinsed in distilled water for 1 min, soaked in ammonium hydroxide for 30 min in the dark, rinsed in distilled water for 1 min again, and placed in Kodak Fix for image capture for 30 min. Subsequently, the slides were rinsed with distilled water for 1 min, with 50% alcohol for 1 min, with 70% alcohol for 1 min, with 95% alcohol for 1 min, twice with 100% alcohol for 5 min, and
with a solution of 100% alcohol and xylene for 15 min. The slides were coverslipped with Permount. The images were acquired with an Olympus eclipse 80i microscope (Olympus, Japan). For quantification, three sections were analyzed for each rat. To examine the morphologies of dendritic spines, individual spine was manually traced and counted, and the length of each spine was measured by MetaMorph software (Universal Imaging, USA).

**Spinal Cord Slice Preparation and Patch-clamp Recordings**

Spinal cord segments (L₄ to L₆) were removed aseptically from the rats (5 to 7 weeks old) under sevoflurane (3.0%) anesthesia and sliced into transverse slices (350 µm) with a vibrating microslicer. Then the slices were incubated at 22 to 25°C in preoxygenated artificial cerebrospinal fluid solution composed of 126 mM NaCl, 2 mM MgCl₂, 3.5 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 2 mM CaCl₂, and 10 mM d-glucose. Subsequently, each slice was transferred into a recording chamber, which was placed on an upright microscope equipped (BX51W1; Olympus, Japan) and continuously perfused with oxygenated artificial cerebrospinal fluid. Individual neurons were identified by a television monitor connected to a low light-sensitive charge-coupled device camera (710M; DVC, USA). Borosilicate glass patch microelectrodes, purchased from the vertical electrode puller (PIP5; HEKA, Germany), were employed for whole-cell patch-clamp recording with the tip openings of 1 to 2 µm and a series resistance of 3 to 5 MΩ. The microelectrodes were filled with intracellular solution (pH 7.3) containing 130 mM KCl, 0.5 mM CaCl₂, 10 mM HEPES, 2 mM MgCl₂, 10 mM EGTA, 2 mM Mg-ATP, and 0.3 mM Na-GTP. AMPA receptor-mediated miniature excitatory postsynaptic currents were isolated in the presence of (2R)-amino-5-phosphonovaleric acid (40 µM), strychnine (2 µM), tetrodotoxin (0.5 µM), and bicusculine (5 µM). The recording data were analyzed using Clampfit 9.0 (Axon Instruments, USA).

**Statistical Analysis**

All statistical analyses were performed with SPSS 18.0 software (SPSS, Inc., USA). For calculating the sample size, a power analysis was implemented on the basis of our preliminary study. The mean paw withdrawal threshold (primary outcome) at baseline (the day before remifentanil exposure) was 19.0 g, whereas the data of the control group (saline group) and three treatment groups (incision, remifentanil, and incision + remifentanil groups) at postoperative 48 h were 18.8 ± 1.3, 11.6 ± 1.8, 12.1 ± 2.4, and 6.9 ± 1.7 g, respectively. Thus, when compared with control group, we expected a difference in means of at least 6.7 g among treatment groups. An *a priori* algorithm was utilized to find the necessary sample size for ANOVA with repeated measures. Therefore, a sample size of five rats/group was required for acquiring accurate and reasonable results. All animals were randomly assigned to experimental conditions, and all data were included for observation and statistics.

The Shapiro–Wilks test was conducted for determining the normality of data distribution, and parametric statistics were applied. The homogeneity of variance was validated by the Levene test. The statistical analyses of behavioral data were performed by two-way ANOVA with Bonferroni post hoc comparisons. The results of Western blot, Golgi staining, and electrophysiologic data were analyzed using one-way ANOVA with Bonferroni post hoc comparisons. *P < 0.05* was considered statistically significant. All data are expressed as means ± SD.

**Results**

**Intraoperative Exposure to Remifentanil Facilitated the Behavioral Hyperalgesia and Upregulated the Spinal Protein Kinase Mζ Activity after Surgery**

Herein, we reported that the paw withdrawal threshold and latency were dramatically and similarly reduced from 2 h to 3 days after incision or remifentanil treatment as compared to the saline rats (all *P < 0.0001*, *n = 7*; fig. 1, A and B), suggesting that mechanical and thermal hyperalgesia caused by either incision or remifentanil was sustained for less than 5 days. Interestingly, remifentanil anesthesia apparently aggravated and prolonged the incision-induced mechanical and thermal hyperalgesia, which was indicated by a significant and long-lasting (more than 7 days) decrease in both the paw withdrawal threshold (*P < 0.0001*) and latency (*P < 0.0001*) after operation (*n = 7*; fig. 1, A and B). By contrast, we did not detect a significant difference in the mean arterial blood pressure during remifentanil anesthesia as compared to the baseline (*P = 0.377*, *n = 7*; Supplemental Digital Content, http://links.lww.com/ALN/B684), indicating that all rats acquired adequate analgesia during surgery. Remifentanil anesthesia–related changes in the spinal dorsal horn are critical for the generation of postoperative opioid-induced hyperalgesia.³³,³⁸,³⁹ Western blot analysis exhibited a rapid (within 2 h) and long-lasting (more than 7 days) increase in the expression of protein kinase Mζ after remifentanil and incision intervention (*P < 0.0001*, *n = 4*; fig. 2, A and B), which was similar to behavioral opioid-induced hyperalgesia. Immediately after synthesis, protein kinase Mζ is minimally active until the phosphorylation that alters the conformation and intensifies the activity, which is required for persistent enhancement of synaptic transmission.³⁹ Intriguingly, the phosphorylation of protein kinase Mζ at Thr410 was rapidly upregulated within 2 h, peaked at 2 days, and continued for more than 7 days in incision-treated rats under remifentanil anesthesia (*P < 0.0001*, *n = 4*; fig. 2, A and B). Moreover, at 2 days postsurgery, when compared with vehicle, both formation and phosphorylation of spinal protein kinase Mζ remarkably increased in rats receiving remifentanil (*P = 0.005* and *P = 0.014*) and incision (*P = 0.039* and *P = 0.038*), especially rats with
PAIN MEDICINE

Fig. 1. Time course of mechanical and thermal hyperalgesia after incision and remifentanil anesthesia. When compared with rats receiving saline, incision and remifentanil significantly decreased paw withdrawal mechanical threshold (A) and paw withdrawal thermal latency (B). Moreover, incision and remifentanil treatment further increased mechanical and thermal hyperalgesia induced by incision. All the data are means ± SD (n = 7) and analyzed by two-way ANOVA with Bonferroni post hoc comparisons. #P < 0.05 versus group saline; *P < 0.05 versus group incision; ^P < 0.05 versus baseline. d = days.

Fig. 2. Time course of protein kinase Mζ (PKMζ) activity, Kalirin-7 expression, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor trafficking in the spinal dorsal horn in remifentanil-induced postoperative hyperalgesia. (A) Representative bands of Western blot for the levels of phosphorylated protein kinase Mζ, pPKMζ (Thr410, lower band, 55 kd), Kalirin-7, and total (t) and membrane (m) GluA1 and GluA2 protein in the spinal dorsal horn. (B–D) When compared with naive rats, the expression and phosphorylation of PKMζ (B), the level of Kalirin-7 (C), and the expression and trafficking of GluA1 (D) were significantly upregulated after incision with remifentanil anesthesia. (E) However, the expression and trafficking of GluA2 were unaltered. All the data are means ± SD (n = 4), and one-way ANOVA with Bonferroni post hoc testing was performed for comparisons between different time points. *P < 0.05; **P < 0.01 versus group naive. d = days.

remifentanil-induced postoperative hyperalgesia (P < 0.0001 and P = 0.001, n = 4; fig. 3, A and B).

Elevation of Spinal Kalirin-7 Expression and AMPA Receptor Trafficking in Postoperative Opioid-induced Hyperalgesia

The Rho GEF Kalirin-7 is strongly implicated in AMPA receptor-mediated excitatory synaptic transmission, revealing a link between Kalirin-7 and AMPA receptor in pathologic conditions.28–30 Because of the role of AMPA receptor in the nociceptive process,13–16 the expression of Kalirin-7 and the trafficking of AMPA receptor in the dorsal horn of both remifentanil- and incision-treated rats was examined. Postoperatively, the opioid-induced hyperalgesia rats demonstrated an abrupt and long-lasting upregulation of Kalirin-7 level, and the expression of GluA1 in both total (t) and membrane (m)
Protein Kinase Mζ Regulates AMPA via Kalirin-7

proteins was considerably elevated (all \( P < 0.0001, n = 4 \); fig. 2, A, C, and D). Moreover, the trafficking of GluA1-containing AMPA receptor from cytosol to surface was also raised, which was indicated by an increase in the ratio of membrane/total GluA1 after incision with remifentanil infusion (\( P = 0.004, n = 4 \); fig. 2, A and D). However, we did not detect any postoperative difference of the membrane GluA2 (\( P = 0.059 \)) and total GluA2 (\( P = 0.196 \)) expression when compared with naive rats (\( n = 4 \); fig. 2, A and E). Intriguingly, the levels of Kalirin-7 expression and GluA1-containing AMPA receptor trafficking in rats receiving either incision (\( P = 0.018 \) and \( P = 0.042 \)) or remifentanil (\( P = 0.002 \) and \( P = 0.037 \)) were also elevated as compared to vehicle; moreover, the highest levels were noted in rats receiving incision and remifentanil treatment. However, the expression and trafficking of GluA2 were unaltered. All the data are means ± SD (\( n = 4 \)) and analyzed by one-way ANOVA with Bonferroni post hoc comparisons. \# \( P < 0.05 \) versus group saline; * \( P < 0.05 \) versus group incision.

Fig. 3. Remifentanil anesthesia further upregulates spinal protein kinase Mζ (PKMζ) activity, Kalirin-7 expression, and \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor trafficking after surgery. (A) Representative bands of Western blot for the levels of phosphorylated protein kinase Mζ (pPKMζ(Thr410), lower band, 55 kd), Kalirin-7, and total (t) and membrane (m) GluA1 and GluA2 protein at postoperative 48 h. (B–D) When compared with saline, incision and remifentanil upregulated the expression and phosphorylation of protein kinase Mζ (B), the level of Kalirin-7 (C), and the expression and trafficking of GluA1 (D). Moreover, the highest levels were noted in rats receiving incision and remifentanil treatment. (E) However, the expression and trafficking of GluA2 were unaltered. All the data are means ± SD (\( n = 4 \)) and analyzed by one-way ANOVA with Bonferroni post hoc comparisons. \# \( P < 0.05 \) versus group saline; * \( P < 0.05 \) versus group incision.

Next, Western blot revealed that the central injection of \( \zeta \)-pseudosubstrate inhibitory peptide dose-dependently blocked the increase in phosphorylation of protein kinase Mζ, inhibited spinal Kalirin-7, and GluA1-containing AMPA receptor overproduction after both remifentanil and incision intervention (all \( P < 0.0001, n = 4 \); fig. 4, E–H). Concurrently, the enhanced trafficking of GluA1-containing AMPA receptor from cytosol to surface in the dorsal horn of rats with postoperative opioid-induced hyperalgesia was abolished by \( \zeta \)-pseudosubstrate inhibitory peptide application (\( P < 0.0001, n = 4 \); fig. 4, E and H).

Increase in Kalirin-7 Expression and AMPA Receptor Trafficking in Opioid-induced Hyperalgesia Was Abolished after Central Protein Kinase Mζ Inhibition

The current behavioral data discovered that intrathecal pretreatment with \( \zeta \)-pseudosubstrate inhibitory peptide (10 and 100 nmol) alleviated and shortened the remifentanil-induced postoperative mechanical and thermal hyperalgesia in a dose-dependent manner (both \( P < 0.0001, n = 7 \); fig. 4, A and B); however, remifentanil intraoperative analgesia was unaltered (\( P = 0.556, n = 7 \); Supplemental Digital Content, http://links.lww.com/ALN/B684). Similarly, either remifentanil or incision decreased the paw withdrawal threshold and paw withdrawal latency that was drastically improved by a single dose of \( \zeta \)-pseudosubstrate inhibitory peptide (100 nmol; fig. 4, C and D). The effect of control scrambled \( \zeta \)-pseudosubstrate inhibitory peptide was also tested, and we did not observe an effective reversal of opioid-induced hyperalgesia (fig. 4, A and B).
Kalirin-7 Deficiency Impaired Remifentanil-induced Postoperative Hyperalgesia and Spinal AMPA Receptor Trafficking

To determine whether Kalirin-7 is required for remifentanil-induced behavioral hyperalgesia and the trafficking of AMPA receptor, lentiviral vector–Kalirin-7–short hairpin RNA was employed to knockdown the expression of spinal Kalirin-7. Western blot showed that in comparison with either saline or lentiviral vector-negative control treated rats, the expression of spinal Kalirin-7 protein was significantly dampened at 2 weeks ($P < 0.003$) and 3 weeks ($P = 0.008$) after intrathecal delivery of lentiviral vector–Kalirin-7–short hairpin RNA ($n = 4$; fig. 5A).

After allowing 2 weeks to establish a stable knockdown, the rats underwent remifentanil and/or incision treatment. All animals receiving gene therapy did not exhibit the defect of locomotor function and normal pain sensitivity (table 1; fig. 5, B and C). However, remifentanil-induced postoperative mechanical and thermal hyperalgesia was markedly attenuated after central Kalirin-7 knockdown (both $P < 0.0001$; fig. 5, B and C). The antihyperalgesia effect was evident 2 h after surgery and persisted for more than 7 days. In addition, either incision- or remifentanil-related hyperalgesia was compromised by this treatment (fig. 5, D and E). Importantly, the expression ($P = 0.95$) and activity ($P = 0.97$) of spinal protein kinase Mζ in rats with remifentanil-incision exposure were unaffected by pretreatment with lentiviral vector–Kalirin-7–short hairpin RNA ($n = 4$; fig. 5, F and G); however, the preadministration of lentiviral vector–Kalirin-7–short hairpin RNA restrained the expression ($< 0.0001$) and trafficking ($< 0.0001$) of GluA1-containing AMPA receptor (fig. 5, E–H).

Intrathecal NASPM Therapy Attenuated the Behavioral Opioid-induced Hyperalgesia and Blocked the Spinal AMPA Receptor Surface Expression

To further verify the effect of AMPA receptor on opioid-induced hyperalgesia, a selective antagonist NASPM$^{33}$ was intrathecally injected to block spinal GluA1-containing AMPA receptor. When compared with rats that underwent incision under the influence of remifentanil anesthesia, the preadministration of NASPM (10 and 100 µg) induced a remarkable and dose-dependent increase in the paw withdrawal threshold and paw withdrawal latency (both $P < 0.0001$, $n = 7$; fig. 6, A and B), without the impairment of remifentanil intraoperative analgesia (Supplemental Digital Content, http://links.lww.com/ALN/B684). Moreover, intrathecal NASPM treatment downregulated the trafficking (but not the total expression) of GluA1-containing AMPA receptor in the dorsal horn of rats with postoperative opioid-induced hyperalgesia ($P < 0.0001$, $n = 4$; fig. 6, C and D). Notably, we discovered that...
NASPM (100 µg) attenuated either remifentanil or incision-induced hyperalgesia; however, no effect was observed on normal pain sensitivity (fig. 6, E and F). Intriguingly, the behavioral tests (n = 7; fig. 6, G and H) also revealed that ζ-pseudosubstrate inhibitory peptide (1 nmol) administration did not alter mechanical (P = 0.516) and thermal hyperalgesia (P = 0.948). Similarly, NASPM (1 µg) had no significant effect on mechanical (P = 0.729) and thermal hyperalgesia (P = 0.099). However, the mechanical (P < 0.0001) and thermal (P < 0.0001) hyperalgesia was remarkably improved by the combination of ζ-pseudosubstrate inhibitory peptide and NASPM with subthreshold doses.

**Kalirin-7 Controls the Formation and Enlargement of Dendritic Spine in the Spinal Dorsal Horn of Opioid-induced Hyperalgesia Rats**

Kalirin-7 is recognized for its central role in the modification of spine plasticity, which is critical for the functional strengthening of excitatory synapses and synaptic trafficking.
of glutamate receptor.\textsuperscript{25–27} Then we employed Golgi staining to detect the changes in the spine morphology. Compared with the saline-treated rats, intraoperative exposure to remifentanil exhibited postoperative spine formation ($P < 0.0001$) and enlargement ($P < 0.0001$) in the dorsal horn (n = 4; fig. 7, A–C). As expected, Kalirin-7 knockdown reversed the increase in spine density ($P < 0.0001$) and areas ($P = 0.001$) in rats with postoperative opioid-induced hyperalgesia (n = 4; fig. 7, A–C). In addition, we found that preadministration of $\zeta$-pseudosubstrate inhibitory peptide (100 nmol), reduced spine formation and enlargement ($P < 0.0001$) due to incision and remifentanil (n = 4; fig. 7, A–C).

### Table 1. Assessment of Locomotor Function after Kalirin-7 Knockdown

<table>
<thead>
<tr>
<th>Group</th>
<th>Placing Reflex</th>
<th>Grasping Reflex</th>
<th>Righting Reflex</th>
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<tbody>
<tr>
<td>Saline</td>
<td>5.4 ± 0.6</td>
<td>5.0 ± 0.5</td>
<td>5.2 ± 0.6</td>
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<tr>
<td>LV–negative control</td>
<td>5.3 ± 0.8</td>
<td>5.2 ± 0.7</td>
<td>5.0 ± 0.6</td>
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<td>LV–Kalirin-7–shRNA</td>
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<td>4.9 ± 0.6</td>
<td>5.1 ± 0.8</td>
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<tr>
<td>$P$ value</td>
<td>0.729</td>
<td>0.721</td>
<td>0.916</td>
</tr>
</tbody>
</table>

Locomotor function was measured at 2 weeks after intrathecal delivery of saline, LV–negative control, and LV–Kalirin-7–shRNA. Every test was repeatedly conducted for six trials, and the counts of each normal reflex were considered as scores for the tests. The results are means ± SD (n = 7) and analyzed by one-way ANOVA with Bonferroni post hoc comparisons. LV = lentiviral vector; shRNA = short hairpin RNA.

### Protein Kinase $M_\zeta$ and Kalirin-7 Modulated the Electrophysiologic Function of Spinal AMPA Receptor in Opioid-induced Hyperalgesia

The whole-cell patch-clamp recordings were employed to confirm the functional regulation of AMPA receptor by protein kinase $M_\zeta$ or Kalirin-7 in opioid-induced hyperalgesia. The representative traces of AMPA receptor-mediated miniature excitatory postsynaptic currents in spinal cord dorsal horn neurons after different interventions are shown in Fig. 7D. The interevent interval of AMPA receptor current was decreased ($P < 0.0001$), whereas the amplitude of AMPA receptor current was increased ($P < 0.0001$) after treatment with 4 nM remifentanil (n = 4; fig. 7, D–F). Strikingly, $\zeta$-pseudosubstrate inhibitory peptide (5 µM) application apparently downregulated the increased amplitude ($P < 0.0001$) and frequency ($P = 0.002$) of AMPA receptor current due to remifentanil exposure (fig. 7, D–F). Additionally, we made the spinal slices from Kalirin-7 knockdown rats at 2 weeks after lentiviral vector–Kalirin-7–short hairpin RNA injection. Then we detected that the Kalirin-7 deficiency substantially inhibited the enhancement of AMPA receptor currents after remifentanil incubation ($P < 0.0001$; fig. 7, D–F).

### Discussion

The principal findings of the current study are as follows: (1) remifentanil anesthesia aggravates incision-related behavioral...
hyperalgesia and facilitates the phosphorylation of protein kinase Mζ, expression of Kalirin-7, trafficking of GluA1-containing AMPA receptor, and the generation of dendritic spine in the spinal dorsal horn; (2) spinal blockade of protein kinase Mζ inhibits the development of postoperative opioid-induced hyperalgesia, the induction of Kalirin-7 and the trafficking of GluA1-containing AMPA receptor in a dose dependent manner; (3) Kalirin-7 deficiency impairs remifentanil-induced hyperalgesia, the expression and trafficking of GluA1-containing AMPA receptor, and spine structural plasticity in the spinal dorsal horn, respectively; and (4) the increase in remifentanil-induced synaptic AMPA receptor currents in the dorsal horn is disrupted by either pharmacologic suppression of protein kinase Mζ or Kalirin-7 knockdown. Collectively, these results recapitulate the importance of the spinal protein kinase Mζ/Kalirin-7/AMPA receptor pathway in signal transduction and synaptic structural and functional plasticity alterations, after remifentanil infusion, which are essential for the generation of central sensitization and pain hypersensitivity (fig. 8).

According to the equivalent dose conversion table between the species, the dose of rat is 6.25 times that of human to achieve the same pharmacodynamic effect. Thus, the human dose is 0.16 µg·kg⁻¹·min⁻¹ converting from the remifentanil dose of 1.0 µg·kg⁻¹·min⁻¹ in rat, which is within the clinically accepted and recommended doses. In this study, all data of intraoperative blood pressure measurements and postoperative nociceptive behavioral tests suggested that remifentanil (1.0 µg·kg⁻¹·min⁻¹) anesthesia caused intraoperative adequate analgesia and postoperative hyperalgesia, which is consistent with previous studies.

Cumulative evidence manifests that central sensitization and nociceptive synaptic plasticity depends on the surface trafficking of GluA1- but not GluA2/3-containing AMPA receptor from cytosol to postsynaptic membrane in the dorsal horn neurons. Herein, we showed that both remifentanil and incision intervention upregulated the expression and trafficking of GluA1-containing AMPA receptor in the spinal dorsal horn. Remarkably, the intraoperative remifentanil anesthesia further elevated the incision-induced expression and trafficking of GluA1-containing AMPA receptor. In contrast, the expression and trafficking of spinal GluA2-containing AMPA receptor remain unaltered in rats with opioid-induced hyperalgesia. To the best of our knowledge, this is

**Fig. 7.** Protein kinase Mζ and Kalirin-7 modulated structural and functional synaptic plasticity in the spinal dorsal horn after remifentanil exposure. Protein kinase Mζ inhibitor ζ-pseudosubstrate inhibitory peptide (ZIP, 100 nmol) and lentiviral vector (LV)-Kalirin-7-short hairpin RNA (K7-shRNA) were injected intrathecally. (A) Representative photomicrographs of spine morphology in the dorsal horn at postoperative 48h were shown. (B, C) The spine density (B) and length (C) were assessed. The results are means ± SD (n = 4) and analyzed by one-way ANOVA with Bonferroni post hoc comparisons. #P < 0.05 versus group saline; *P < 0.05 versus group incision + remifentanil. (D) Representative traces of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor–mediated miniature excitatory postsynaptic currents in the dorsal horn neurons of spinal cord slices after remifentanil incubation (4nM) and the effects of ζ-pseudosubstrate inhibitory peptide (ZIP, 5 µM) and Kalirin-7–short hairpin RNA. (E, F) The amplitude (E) and interevent interval (F) of miniature excitatory postsynaptic currents in the spinal dorsal horn neurons under different conditions were evaluated. The results are means ± SD (n = 4) and analyzed by one-way ANOVA with Bonferroni post hoc comparisons. #P < 0.05 versus group saline; *P < 0.05 versus group remifentanil.
the first study in which the pretreatment with an antagonist to GluA1 ameliorates remifentanil-induced postoperative hyperalgesia and reverses the increased trafficking of spinal GluA1-containing AMPA receptor in a dose-dependent manner. Our results indicated that spinal upregulation of AMPA receptor GluA1 trafficking, after remifentanil exposure, is a critical step in the development of opioid-induced hyperalgesia (fig. 8).

Most studies that support a role for protein kinase Mζ in the maintenance of long-term potentiation have used protein kinase Mζ pharmacologic inhibitor ζ-pseudosubstrate inhibitory peptide but recent studies reported that the absence of protein kinase Mζ did not impair normal synaptic transmission and normal behaviors in mice and that ζ-pseudosubstrate inhibitory peptide could eliminate reward memory even when protein kinase Mζ is not present, which has raised concerns regarding the specificity of ζ-inhibitory pseudosubstrate peptide. Nevertheless, mice lacking both proteins of protein kinase Cζ and protein kinase Mζ were observed to display reduced anxiety-like behavior. Moreover, intracellular protein kinase Mζ perfusion has been reported to facilitate synaptic transmission by increasing the number of synaptic AMPA receptors. In addition, protein kinase Mζ overexpression has been shown to enhance memory for conditioned taste aversion, suggesting that protein kinase Mζ may regulate synaptic plasticity under certain conditions or in select neuronal regions. Recently, we reported that the expression of atypical protein kinase Cs (protein kinase Mζ and protein kinase Cζ but not protein kinase Cλ/τ) in the spinal dorsal horn is increased after remifentanil infusion and that protein kinase Mζ inhibitor ζ-pseudosubstrate inhibitor, but not other protein kinase C inhibitor NPC-15437, reliably alleviated remifentanil infusion-related hyperalgesia, thereby suggesting the implication of protein kinase Mζ in opioid-induced hyperalgesia. The present study demonstrated that remifentanil anesthesia further upregulated the incision-related increase in the expression and activity of protein kinase Mζ. Furthermore, after the intervention of ζ-pseudosubstrate inhibitory peptide, the dose-dependent prevention of postoperative hyperalgesic behavior, the suppression of protein kinase Mζ activity (but not expression), the reversal of GluA1-containing AMPAR trafficking out of the synapse, and the decrease in AMPA receptor-mediated currents in opioid-induced hyperalgesia were observed in vivo and in vitro. To further verify the involvement of protein kinase Mζ in GluA1-containing AMPA receptor in the mechanism of opioid-induced hyperalgesia, protein kinase Mζ inhibitor and GluA1 antagonist

Fig. 8. Schematic illustration of proposed protein kinase Mζ (PKMζ)–Kalirin-7–α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) signaling in remifentanil-induced postoperative hyperalgesia. Glutamate–N-methyl-D-aspartate receptor (NMDAR) activation after remifentanil exposure causes cellular calcium influx, promoting protein kinase Mζ production and phosphorylation. Activated protein kinase Mζ results in Kalirin-7 overexpression, which triggers structural and functional modifications of central excitatory synapses. Formation and enlargement of dendritic spine, and GluA1-containing AMPAR trafficking from the intracellular pool to surface pool contribute to the development of behavioral hyperalgesia. Thus, targeting protein kinase Mζ/Kalirin-7/GluA1-containing AMPAR pathway may offer the possibilities for the prevention of hyperalgesia. CaMKII = Ca²⁺/calmodulin-dependent protein kinase II.
with subthreshold doses were utilized. Strikingly, this is the first report stating that either protein kinase Mζ inhibitor or GluA1 antagonist with subthreshold dose does not impair the behavioral hyperalgesia; however, these phenomena are reversed by their coadministration with the subthreshold dose. Taken together, these results clarified that spinal protein kinase Mζ modulates the trafficking and function of GluA1-containing AMPA receptor, leading to the functional modifications of excitatory synapses and remifentanil-induced postoperative hyperalgesia (fig. 8), thereby indicating the benefits of this pharmacologic inhibition of protein kinase Mζ as promising for therapeutic translation. Nevertheless, the specific interaction between them after remifentanil treatment is yet to be explored.

Intriguingly, according to the literature, rapid structural modifications of central excitatory synapses and altered synaptic functional plasticity in neurodevelopmental and psychiatric disorders are tightly correlated. The dendritic spine structure is critical for synapse function. Kalirin-7 is mostly enriched in dendritic spines of neurons, where it controls spine morphogenesis and the delivery of GluA1 into spines. This is the first study demonstrating that incision, under remifentanil anesthesia, causes a dramatic and time-dependent increase in the levels of spinal Kalirin-7 expression, which is in agreement with the time course of behavioral hyperalgesia. To investigate the regulatory role of spinal Kalirin-7 in remifentanil-induced postsurgical hyperalgesia, intrathecal administration of lentiviral vector–Kalirin-7–short hairpin RNA is engaged in the current model of opioid-induced hyperalgesia. As expected, Kalirin-7 deficiency successfully alleviated the mechanical and thermal hyperalgesia postoperatively, blocked the trafficking and currents of GluA1-containing AMPA receptor, and downregulated the generation and enlargement of the spine in the spinal dorsal horn due to remifentanil anesthesia. This evidence strongly suggested that Kalirin-7 is crucial for the generation and maintenance of structure and function of excitatory synapses during plasticity in the pathogenesis of opioid-induced hyperalgesia (fig. 8). However, the specific mechanism underlying Kalirin-7–regulated activation of AMPA receptor in opioid-induced hyperalgesia necessitates further studies.

Next, given the similar modulation of AMPA receptor trafficking and synaptic plasticity by protein kinase Mζ and Kalirin-7 in the current study and recent literature, we tested the hypothesis that protein kinase Mζ correlates with Kalirin-7 in remifentanil-induced postoperative hyperalgesia. Herein, we elucidated that central protein kinase Mζ inhibition reduced the overproduction of Kalirin-7 in the dorsal horn of rats with postoperative opioid-induced hyperalgesia. On the other hand, the expression and phosphorylation of protein kinase Mζ after both remifentanil and incision treatment was unaltered by Kalirin-7 knockdown, thereby suggesting that Kalirin-7 is a downstream target of protein kinase Mζ and is required for the enhancement of protein kinase Mζ-dependent AMPA receptor trafficking and nociceptive synaptic transmission (fig. 8). These findings also render the possibility of targeting Kalirin-7 for the prevention of opioid-induced hyperalgesia and substantial benefit to patients receiving opioid therapy.

Previous studies have proposed that NR2B-containing NMDA receptor–related central sensitization plays a fundamental role in the initiation of opioid-induced hyperalgesia. We further found that selective NR2B-containing NMDA receptor antagonist is sufficient to abrogate opioid-induced hyperalgesia and protein kinase Mζ activation (fig. 8). Recently, µ-opioid receptor expressed by primary afferent nociceptors mediates opioid-induced spinal long-term potentiation at nociceptor synapses is demonstrated to be a crucial contributor to opioid-induced hyperalgesia. However, whether the increase of spinal protein kinase Mζ activity after remifentanil exposure is initiated by peripheral µ-opioid receptor requires further investigation. In addition, phosphorylated extrasynaptic signal-regulated kinase is important for the maintenance of central nociceptive sensitization and long-term neuronal plasticity via increasing the activity of NMDA receptor and AMPA receptor in dorsal horn neurons. However, the connection between phosphorylated extrasynaptic signal-regulated kinase and AMPA receptor in the model of opioid-induced hyperalgesia is yet to be clarified.

In summary, aberrant protein kinase Mζ-dependent trafficking of AMPA receptor and synaptic plasticity via Kalirin-7 contributes to remifentanil-induced postoperative hyperalgesia. The pharmacologic intervention of protein kinase Mζ/Kalirin-7/GluA1-containing AMPA receptor pathway protects against the development of hyperalgesia. This hypothesis could be extended to other nociceptive states associated with protein kinase Mζ signaling and might expedite the development of novel therapies for the treatment of pathologic pain.

Support for the enhancement of protein kinase Mζ-dependent AMPA receptor trafficking and nociceptive synaptic transmission (fig. 8). These findings also render the possibility of targeting Kalirin-7 for the prevention of opioid-induced hyperalgesia and substantial benefit to patients receiving opioid therapy.

Competing Interests
The authors declare no competing interests.

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Zhang et al.


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Protein Kinase Mζ Regulates AMPA via Kalirin-7


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