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Spinal NLRP3 inflammasome activation mediates IL-1β release and contributes to remifentanil-induced postoperative hyperalgesia by regulating NMDA receptor NR1 subunit phosphorylation and GLT-1 expression in rats

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Abstract

Background: Trafficking and activation of N-methyl-D-aspartate (NMDA) receptors play an important role in initiating and maintaining postoperative remifentanil-induced hyperalgesia (RIH). Activation of the NOD-like receptor protein 3 (NLRP3) inflammasome has been linked to the development of inflammatory and neuropathic pain. We hypothesized that activation of NLRP3 inflammasome mediates IL-1β release and contributes to RIH in rats by increasing NMDA receptor NR1 (NR1) subunit phosphorylation and decreasing glutamate transporter-1 (GLT-1) expression.

Methods: Acute exposure to remifentanil (1.2 μg/kg/min for 60 min) was used to establish RIH in rats. Thermal and mechanical hyperalgesia were tested at baseline (24 h before remifentanil infusion) and 2, 6, 24, and 48 h after remifentanil infusion. The levels of IL-1β, GLT-1, phosphorylated NR1 (phospho-NR1), and NLRP3 inflammasome activation indicators [NLRP3, Toll-like receptor 4 (TLR4), P2X purinoceptor 7 (P2X7R), and caspase-1] were measured after the last behavioral test. A selective IL-1β inhibitor (IL-1β inhibitor antagonist; IL-1ra) or three different selective NLRP3 inflammasome activation inhibitors [(+)-naloxone (a TLR4 inhibitor), A438079 (a P2X7R inhibitor), or ac-YVADcmk (a caspase-1 inhibitor)] were intrathecally administered immediately before remifentanil infusion into rats.

Results: Remifentanil induced significant postoperative hyperalgesia, increased IL-1β and phospho-NR1 levels and activated the NLRP3 inflammasome by increasing TLR4, P2X7R, NLRP3, and caspase-1 expression, but it decreased GLT-1 expression in the L4-L6 spinal cord segments of rats, which was markedly improved by intrathecal administration of IL-1ra, (+)-naloxone, A438079, or ac-YVADcmk.

Conclusion: NLRP3 inflammasome activation mediates IL-1β release and contributes to RIH in rats by inducing NMDA receptor NR1 subunit phosphorylation and decreasing GLT-1 expression. Inhibiting the activation of the NLRP3 inflammasome may be an effective treatment for RIH.

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Keywords
Remifentanil, postoperative hyperalgesia, NOD-like receptor protein 3 inflammasome, N-methylessentialate receptors, glutamate transporter-1

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Introduction

Opioids are the primary drugs used for anesthesia in the clinic. However, the onset of opioid-induced hyperalgesia (OIH) is associated with opioids. OIH is defined as increased pain from noxious stimuli.[1,2] Remifentanil is an ultra-short-acting opioid often used as an analgesic for general anesthesia and other medical fields.[3] Numerous studies focusing on postoperative remifentanil-induced hyperalgesia (RIH) have been published.[4–6] Furthermore, our previous studies have reported that the intraoperative use of remifentanil may increase postoperative pain scores and the need for opioids.[3,7–9] However, the mechanisms underlying RIH are unclear.

Emerging evidence has shown that the activation of the NOD-like receptor protein 3 (NLRP3) inflammasome contributes to the development of inflammatory and neuropathic pain.[10,11] As a series studies reported by Grace, et al.,[10–13] the activation of the NLRP3 inflammasome requires a series of protein complexes. The role of Toll-like receptor 4 (TLR4) in OIH and hyperalgesia priming has been proven. The TLR4 increase has been shown to enhance the expression of NLRP3 and pro-IL-1β, which contributes to the activation of the NLRP3 inflammasome.[11,13] As the secondary signaling, the release of danger-associated molecular patterns (DAMPs) induced by remifentanil could increase the purinergic receptor P2X purinoceptor 7 (P2X7R) expression, lead to the association of NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and Caspase-1 to be the NLRP3 inflammasome complex, and then activates IL-1β, which is a gatekeeper of inflammation.[10,12] Moreover, Zhang et al.[14] have reported that IL-1β receptor type I (IL-1βRI) and NMDA receptor NR1 (NR1) subunit are co-localized in spinal neurons, and the increase in IL-1β may directly act on NMDA receptor-containing neurons and influence NR1 phosphorylation. Studies have also demonstrated that IL-1β can inhibit astrocyte glutamate uptake through the downregulation of glutamate transporter-1 (GLT-1), leading to an increase in extracellular glutamate release. Accumulated extracellular glutamate has been shown to enhance NR1 phosphorylation.[14,15]

NMDA receptors are ionotropic glutamate receptors that contribute to excitatory synaptic transmission.[8] NMDA NR1, NR2, and NR3 are the three NMDA receptor subunits. The trafficking and activation of NMDA receptor NR1 subunits play a pivotal role in developing and maintaining RIH.[16] The trafficking of NMDA receptor NR1 subunits depends on the phosphorylation of NMDA receptor NR1 subunits.[17] Moreover, GLT-1 is primarily responsible for glutamate clearance in the spinal cord.[15] Repeated opioid administration reduces spinal GLT-1 expression.[18] Glutamate is the primary excitatory neurotransmitter in the central nervous system that initiates rapid signal transmission in the synapse before its reuptake into the surrounding glia, and specifically, astrocytes. GLT-1 is the primary transporter that takes up synaptic glutamate to maintain optimal extracellular glutamic levels, thus preventing accumulation in the synaptic cleft and the consequent excitotoxicity. Growing evidence has shown that excitotoxicity is associated with various neurological disorders, including OIH.[19] While the mechanisms of RIH are not well understood, the downregulation of GLT-1 may play a significant role in RIH.

Inspired by articles published by Grace, et al.[10–13] and Zhang, et al.,[14] the present study aimed to explore the mechanisms underlying RIH. We hypothesized that NLRP3 inflammasome activation mediates IL-1β release and contributes to RIH by regulating phospho-NR1 and GLT-1 expression. The inhibition of either NLRP3 inflammasome activation or IL-1β release may be effective as a novel strategy for treating RIH.

Materials and methods

Animals

In total, 80 Adult male Sprague-Dawley rats (weight, 240–260 g; Laboratory Animal Center of the Military Medical Science Academy of the People's Liberation Army) were used in the present study. The animals were fed with standard food and sufficient water in cages under a controllable circumstance (temperature, 20–23°C; humidity 55–65%; 12-h light-dark cycle). The Experimental Animals approved all experimental protocols Ethical Committee of Tianjin Medical University (approval no. ZYY-2018-DW-09) and reported using the Animals in Research: Reporting In Vivo Experiments 2010 guidelines.[20] If infection at the surgical site of rats was suspected, as evidenced by wound splitting or weight loss (>20%), cachexia, or difficulty eating, drinking, or walking, the rat was euthanized by 100% carbon dioxide inhalation. None of the animals was euthanized for any of the above reasons in the present study.

Medication

Remifentanil hydrochloride was purchased from Guangzhou Renfu Medical Equipment Co., Ltd, dissolved in saline (NaCl 0.9%), and infused intravenously at a rate of

1.2 μg/kg/min for 60 min, at our previously reported dose.[7] Controls received the same volume of saline under identical conditions. Sevoflurane was purchased from Maruishi Pharmaceutical Co.; I IL-1β inhibitor antagonist (IL-1ra) from Abcam, A438079 from Tocris, and (+)-Naloxone, ac-YVAD-cmk, and DMSO from Merck KGaA. For intrathecal drug delivery, the catheters were preloaded with drugs at the distal end in a total volume and delivered over 20–30 s from when the catheter was in position. Intrathecal doses were as follows: IL-1ra, 100 μg in 10 μL; (+)-naloxone, 1,200 μg in 10 μL; A438079, 600 ng in 10 μL; and ac-YVADcmk, 20 μg in 10 μL, based on the dose reported by Grace, et al.[10,12] A total of 0.15 μl DMSO dissolved in 10 μl saline was used as a vehicle in each group.

Intrathecal catheter implantation

Rats were anesthetized by continuous inhalation of 3% sevoflurane and 60% oxygen. The intrathecal catheter implantation was performed as previously described.[10,21] Briefly, an intrathecal polyethylene catheter was inserted into the subarachnoid space at the level of the lumbar enlargement segments of the spinal cord through an incision at the atlantooccipital membrane. Some lidocaine cream was applied after the procedure to alleviate the ache. After a 1-week recovery, rats that showed signs of neurologic impairment, according to locomotor function testing, were euthanized.

Experimental design

Experiment one. Behavioural and molecular biological changes following remifentanil infusion. The animals were randomly divided into two groups (n = 8/group; all the animals were used for behavioral tests, and 4/8 animals were randomly selected for tissue analysis right after the last behavioral tests): Normal saline and Remifentanil groups. The animals in the Remifentanil group were infused intravenously with remifentanil hydrochloride (which was dissolved in normal saline) at a rate of 1.2 μg/kg/min for 60 min. The animals in the Normal saline group received the same volume of saline under identical conditions. The withdrawal threshold and latency to mechanical and thermal stimulation, respectively, were evaluated at the baseline (24 h before remifentanil infusion) and 2, 6, 24, and 48 h after remifentanil infusion. The L4-L6 segments of the spinal cord were collected after the last behavioral test to measure the expression of IL-1β, as well as that of GLT-1, phospho-NR1, and the indicators of NLRP3 inflammasome activation (TLR4, P2X7R, NLRP3, and caspase-1; Figure 1).

Experiment two. Role of IL-1β in the development of RIH. The animals were randomly divided into three groups (n=8/group; all the animals were used for behavioral tests, and 4/8 animals were randomly selected for tissue analysis after the last behavioral tests): The Normal saline + Vehicle, Remifentanil + Vehicle, and Remifentanil + IL-1ra groups. Remifentanil or normal saline was administered intravenously as expressed in experiment one. IL-1ra (a selective IL-1β inhibitor; 100 μg dissolved in 10 μl vehicle) was intrathecally administered into the rats of the Remifentanil + IL-1ra group immediately before remifentanil infusion. The animals in the Normal saline + Vehicle and Remifentanil + Vehicle groups received the same volume of the vehicle (0.15 μl DMSO dissolved in 10 μl saline) under identical conditions. The withdrawal threshold and latency to mechanical and thermal stimulation, respectively, were measured at the baseline (24 h before remifentanil infusion) and 2, 6, 24, and 48 h after remifentanil infusion. The L4-L6 segments of the spinal cord were collected after the last behavioral test to measure the levels of GLT-1 and phospho-NR1 (Figure 1).

Experiment three. Role of NLRP3 inflammasome activation in the development of RIH. The animals were randomly divided into five groups (n=8/group; all animals were used for behavioral tests, and 4/8 animals were randomly selected for tissue analysis right after the last behavioral tests): The Normal saline + Vehicle, Remifentanil + Vehicle, Remifentanil + (+)-naloxone, Remifentanil + A438079 and Remifentanil + ac-YVAD-cmk groups. Remifentanil or normal saline was administered intravenously, as expressed in experiment one. Three different selective NLRP3 inflammasome activation inhibitors, (+)-Naloxone (a TLR4 inhibitor, 1,200 μg dissolved in 10 μl vehicle), A438079 (a P2X7R inhibitor, 600 ng dissolved in 10 μl vehicle) and ac-YVAD-cmk (a caspase-1 inhibitor, 20 μg dissolved in 10 μl vehicle) were intrathecally administered into the rats of the Remifentanil + (+)-naloxone, Remifentanil + A438079 and Remifentanil + ac-YVAD-cmk groups, respectively, immediately before remifentanil infusion. The animals in the Normal saline + Vehicle and Remifentanil + Vehicle groups received the same volume of the vehicle (0.15 μl DMSO dissolved in 10 μl saline) under identical conditions. The withdrawal threshold and withdrawal latency to mechanical and thermal stimulation, respectively, were measured at the baseline (24 h before remifentanil infusion) and 2, 6, 24, and 48 h after remifentanil infusion. The L4-L6 segments of the spinal cord were collected after the last behavioral test to measure the levels of GLT-1 and phospho-NR1 (as shown in Figure 1).

Behavioral tests

Mechanical hyperalgesia. An electronic Von Frey filament (cat. no, BSEVF3; Harvard Apparatus) was used to measure the paw withdrawal threshold (PWT) by two blinded experimenters. One experimenter put the numbered animals into wire chambers (20x20x20 cm) with a grid bottom. The other one, responsible for measuring the mechanical

threshold, was blinded to the animals' treatment. The animals were allowed to acclimatize for 1 h before testing. The filament was applied vertically to the area adjacent to the wound of the left hind paw with increasing pressure. The PWT was defined as the pressure (g) at which the rat flinched, shook, or licked its paw. The test was repeated three times at 5-min intervals. The mean of the three trials was regarded as the PWT. A cut-off pressure of 60 g was used to avoid tissue damage.

Thermal hyperalgesia

Intellective hot plate equipment (cat. no. YLS-6 B; Shanghai Zhenghua Medical Equipment Co., Ltd) was used to measure the paw withdrawal latency (PWL) by two blinded experimenters. One experimenter numbered all the animals in the different groups, and the other, who was blinded to the animal grouping, measured the PWL. Animals were allowed to acclimate to the environment for 1 h before testing by placing a hot plate (50°C). The PWL was defined as the time (sec) when the rat showed a positive response (a clear paw withdrawal). The test was repeated three times at 10-min intervals. The mean of the three trials was regarded as the PWL. A cut-off time of 30 s was used to avoid tissue damage.

Tissue preparation

Animals were anesthetized with 2% pentobarbital (130 mg/kg) via intraperitoneal injection. The L4-6 spinal cord was rapidly removed and frozen in liquid nitrogen.

Quantification of mRNA via reverse transcription-quantitative PCR (RT-qPCR)

The levels of IL-1β mRNA in the spinal cord were determined following the last behavioral test. The L4-6 spinal cord was removed and frozen in liquid nitrogen. Total RNA was extracted with TRIzol® (Thermo Fisher Scientific, Inc.). Next, total RNA was transcribed into cDNA with a cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The expression of IL-1β mRNA was determined by RT-qPCR using SYBR Green PCR Master Mix (Roche Diagnostics GmbH). GAPDH was used as an internal control. Each test was run in triplicate. The primers of IL-1β and GAPDH were designed and synthesized by Beijing SBS Genetech Co., Ltd: IL-1β, 5'-GAGGCTCCATCTCCAAGGAC-3' forward and 3'-ACTGTGTGTGACAGGTTGGA-5' reverse; GAPDH, 5'-TGATGGGTGTGAACCACGAG-3' forward and 3'-ATCACGCCACAGCTTTCCAG-5' reverse. IL-1β gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method.[22]

Western blotting

The tissues from the L4-6 spinal cord were removed after the last behavioral test and frozen in liquid nitrogen. Tissues were homogenized in an SDS sample buffer containing proteinase inhibitors. The homogenate was centrifuged at 4°C (12,000 r/min, 10 min), and the supernatant liquor was pipette as the total protein. The protein content was determined using the bicinchoninic acid assay method. Before the proteins were transferred to PVDF membranes, they were separated on an SDS-PAGE gel. The membranes were blocked with 5% non-fat milk for 1 h on the shaker and subsequently incubated with primary antibody rabbit anti-phospho-NR1 (cat. no, ab75680; dilution, 1:500; Abcam), rabbit anti-GLT-1 (cat. no, ab106289; dilution, 1:1,000; Abcam), rabbit anti-TLR4 (cat. no, ab13867; dilution, 1:1,200; Abcam), rabbit anti-P2X7R (cat. no, ab48871; dilution, 1:1,000; Abcam), rabbit anti-NLRP3 (cat. no, ab214185; dilution, 1:1,000; Abcam) and rabbit anti-caspase-1 antibodies (cat. no, ab62698; dilution, 1:1,000; Abcam) overnight at 4°C. The membranes were washed with TBST buffer for 30 min and incubated with an HRP-conjugated goat anti-rabbit IgG secondary antibody (dilution, 1:5,000; cat. no, ab7090; Abcam) for 1 h at room temperature. The proteins were visualized by enhanced chemiluminescence detection (EMD Millipore) using a Bio-Rad GS-700 imaging system with software (Bio-Rad Laboratories, Inc.). β-Actin was used as a loading control. The results are expressed as the ratio to β-actin immunoreactivity.

ELISA

According to the manufacturers' instructions, the level of IL-1β in the rat L4-6 ipsilateral dorsal spinal cord was detected using ELISA kits (Abcam). We determined the optical density of each well using a Fluorescence Plate Reader (Medical Device, San Jose, CA) at 450 nm. The concentrations of IL-1β were measured in duplicates.

Statistical analysis

All data are expressed as the mean ± SD. A Shapiro-Wilk normality test was used to test whether the data were normally distributed. An unpaired Student's t-test was used for values with normal distribution, while the Mann-Whitney test was used for values with non-normal distribution to analyze differences between two groups. One-way ANVOA with Bonferroni comparison was also used to analyze the interactions among various groups. $p < 0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS statistical software (version 16.0; SPSS software) and GraphPad Prism software (version 5.0; GraphPad Software, Inc.).

Results

Remifentanil amplifies postoperative hyperalgesia, enhances IL-1β and phospho-NR1 expression, and reduces GLT-1 levels in rats

To assess whether remifentanil could induce hyperalgesia, remifentanil (1.2 μg/kg/min) or saline was intravenously infused for 60 min. The PWT to von Frey filament stimulation and PWL to thermal stimulation were evaluated at the baseline (24 h before the incision) and 2, 6, 24, and 48 h after remifentanil infusion. Compared with the baseline, the animals in the Normal saline group did not exhibit significant changes in neither PWT nor PWL; $p > 0.05$ vs. Normal saline). However, compared with the Normal saline group, remifentanil infusion induced a significant decrease in the PWT and PWL levels in the left paw of rats at 2–48 h postoperatively ($p < 0.05$ vs. Normal saline; Figure 2).

Following the behavioral tests, the L4-L6 spinal cord segments were collected. Compared with the Normal saline group, the mRNA and protein levels of IL-1β in animals that had undergone remifentanil administration were significantly increased ($p < 0.05$ vs. Normal saline). Moreover, the increased phospho-NR1 and decreased GLT-1 protein levels were identified in spinal cords from the Remifentanil group compared with that of the Normal saline group ($p < 0.05$ vs. Normal saline; Figure 3).

Inhibition of IL-1β can alleviate RIH by regulating phospho-NR1 and GLT-1 expression in rats

To verify the role of IL-1β in the development and maintenance of RIH, a selective IL-1ra was intrathecally administered immediately before the remifentanil infusion in rats. As compared with the animals in the Normal saline + Vehicle group, the animals in the Remifentanil + vehicle group developed mechanical and thermal hyperalgesia, which were reflected by a decrease in the PWT and PWL levels ($p < 0.05$ vs. Normal saline + Vehicle) at 2, 6, 24 and 48 h after remifentanil infusion. However, the PWT and PWL levels in the Remifentanil + IL-1ra group were increased at 2 and 6 h, compared with the Remifentanil + Vehicle group ($p < 0.05$ vs. Remifentanil + Vehicle), suggesting that the inhibition of IL-1β could alleviate the postoperative hyperalgesia induced by remifentanil.

In addition, as compared with the Normal saline + Vehicle group, the Remifentanil + Vehicle group increased phospho-NR1 but decreased GLT-1 expression ($p < 0.05$ vs. Normal saline + Vehicle), which were significantly reversed by pretreatment with IL-1ra ($p < 0.05$ vs. Remifentanil + IL-1ra; Figure 4).

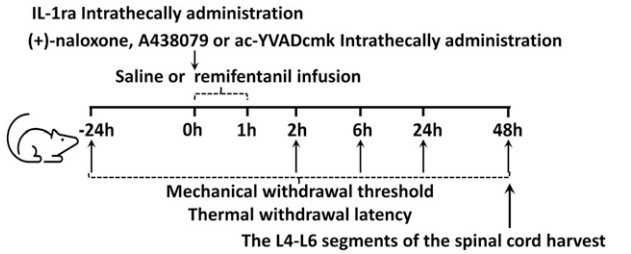

Figure 1. Experimental design. Adult male Sprague-Dawley rats (240-260 g) were subjected to normal saline or remifentanil infusion. Animals in the Remifentanil group were infused intravenously with remifentanil hydrochloride (dissolved in normal saline) at a rate of 1.2 μg/kg/min for 60 min. Animals in the Normal saline group received the same volume of saline under identical conditions. The withdrawal threshold and latency to mechanical and thermal stimulation, respectively, were evaluated at the baseline (24 h before remifentanil infusion) and 2, 6, 24, and 48 h after remifentanil infusion. The L4-L6 segments of the spinal cord were collected after the last behavioral test. To verify the roles of IL-1β and NLRP3 inflammasome activation in the development and maintenance of RIH, a selective IL-1ra or three different selective NLRP3 inflammasome activation inhibitors [(+)-naloxone (a TLR4 inhibitor), A438079 (a P2X7R inhibitor), or ac-YVADcmk (a caspase-1 inhibitor)] were intrathecally administered immediately before remifentanil infusion in rats. RIH, remifentanil-induced hyperalgesia; IL-1ra, IL-1β inhibitor; NLRP3, NOD-like receptor protein 3; P2X7R, P2X purinoceptor 7.

NLRP3 inflammasome inactivation may mitigate RIH by regulating IL-1β-dependent phospho-NR1 and GLT-1 expression in rats

Evidence has shown that the activation of the NLRP3 inflammasome contributes to the development of inflammatory and neuropathic pain (10). As reported above, NLRP3 inflammasome activation requires a series of protein complexes, such as TLR4, P2X7R, and caspase-1. As shown in Figure 5, there was a higher protein expression of NLRP3, TLR4, P2X7R, and caspase-1 in the spinal cord of animals from the Remifentanil group, compared with that of the Normal saline group ($p < 0.05$ vs. Normal saline). The data indicated that remifentanil infusion might enhance the TLR4, P2X7R, and caspase-1 expression, activating the NLRP3 inflammasome.

To test the critical role of NLRP3 inflammasome activation in the development and maintenance of RIH, three different selective NLRP3 inflammasome activation antagonists, including (+)-Naloxone (a TLR4 inhibitor), A438079 (a P2X7R inhibitor), and ac-YVAD-cmk (a caspase-1 inhibitor), were intrathecally administered respectively into the rats immediately before remifentanil infusion. As compared with the animals in the Normal saline + Vehicle group, the animals in the Remifentanil + vehicle group developed mechanical and thermal hyperalgesia, which were reflected by a decrease in the PWT and PWL levels ($p < 0.05$ vs. Normal saline + Vehicle) at 2, 6, 24 and 48 h after remifentanil infusion. However, the PWT and PWL levels in

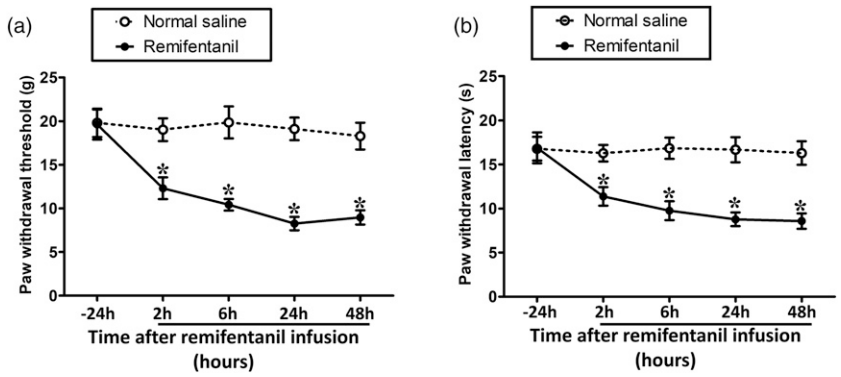

Figure 2. Behavioral changes after remifentanil infusion in rats. Under 3% sevoflurane anesthesia, remifentanil (1.2 μg/kg/min) or saline was intravenously infused for 60 min. The (A) PWT to von Frey filament stimulation and (B) PWL to thermal stimulation were evaluated 24 h before the incision and 2, 6, 24, and 48 h after the remifentanil infusion. The data are presented as the mean ± SD (n = 8/group). *P < 0.05 vs. Normal saline group. PWL, paw withdrawal latency; PWT, paw withdrawal threshold.

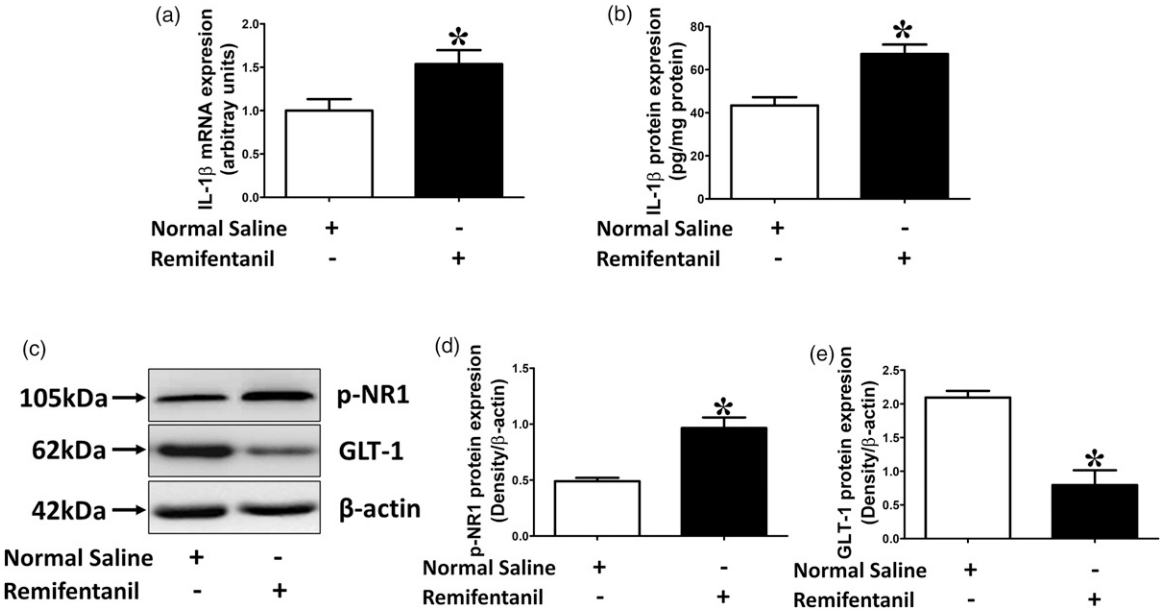

Figure 3. IL-1β, p-NR1 and GLT-1 expression after remifentanil infusion in rats. The L4-L6 segments of the spinal cord were harvested right after the last behavioral test. (A) mRNA and (B) protein levels of IL-1β were detected by RT-qPCR and ELISA, respectively. (C) The p-NR1 and GLT-1 expression were measured by western blotting, and quantitative analysis of (D) p-NR1 and (E) GLT-1 are shown as the ratio of protein relative density to β-actin. The data are presented as the mean ± SD (n = 4/group). *P < 0.05 vs. Normal saline group. RT-qPCR, reverse quantitative transcription PCR; p-NR1, phospho-NMDA receptor NR1; GLT-1, glutamate transporter-1.

the Remifentanil + (+)-Naloxone, Remifentanil + A438079, and Remifentanil + ac-YVAD-cmk groups were significantly increased at 6, 24, and 48 h, as compared with the Remifentanil + Vehicle group ($p < 0.05$ vs. Remifentanil + Vehicle; Figure 6), suggesting that the inhibition of TLR4, P2X7R or caspase-1 expression could alleviate the postoperative hyperalgesia induced by remifentanil.

Furthermore, compared with the Normal saline + Vehicle group, the Remifentanil + Vehicle group increased the mRNA and protein levels of IL-1βand the phospho-NR1 level but decreased GLT-1 ($p < 0.05$ vs. Normal saline + Vehicle). However, pretreatment with (+)-Naloxone, A438079, or ac-YVAD-cmk could induce a higher phospho-NR1 and lower GLT-1 expression, as compared with that in the Remifentanil + Vehicle group ($p < 0.05$ vs. Remifentanil + IL-1ra; Figure 6).

Discussion

Postoperative RIH presents a challenge to anesthesiologists.[23] Our previous studies have already reported that the intraoperative use of remifentanil may increase postoperative pain scores and the need for opioids.[3,7–9] However, its underlying mechanisms remain unclear. In 2016, Grace, et al.

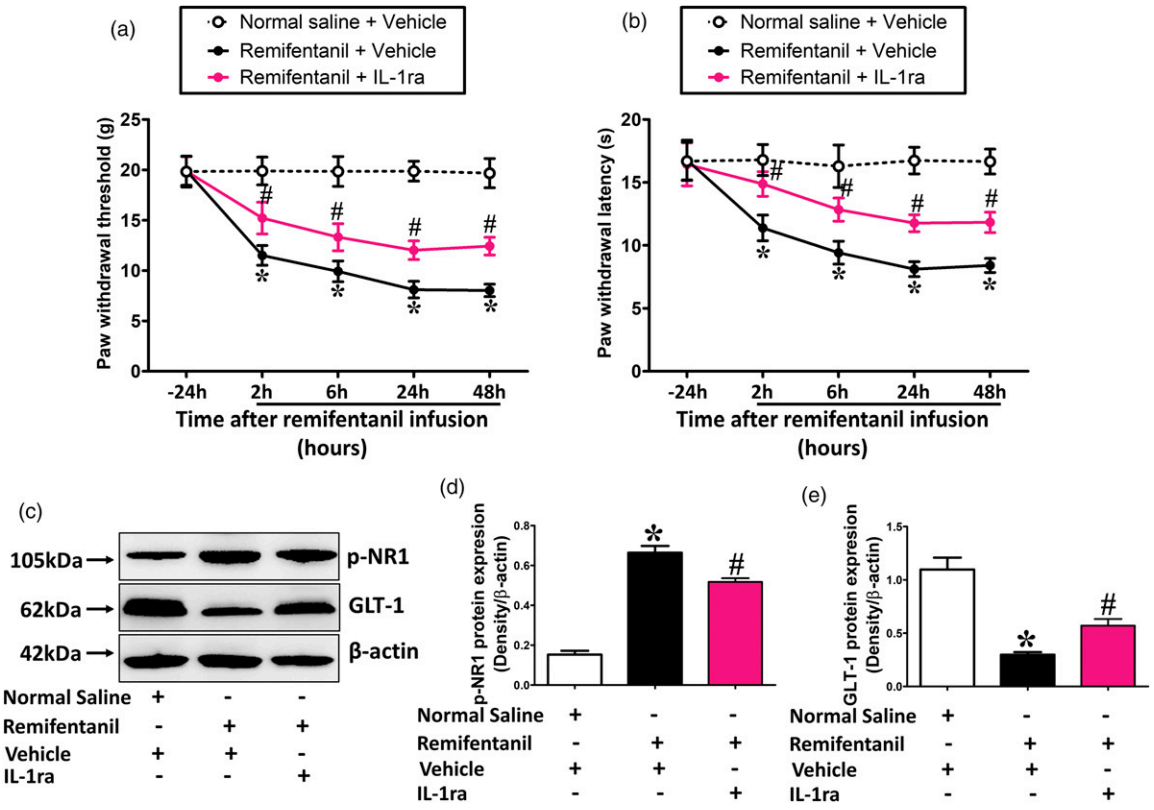

Figure 4. IL-1ra on behavioral changes, p-NR1 and GLT-1 expression after remifentanil infusion in rats. IL-1ra (a selective IL-1β inhibitor; 100 μg dissolved in 10 μl vehicle) or vehicle (0.15 μl DMSO dissolved in 10 μl saline) was intrathecally administered into the rat before remifentanil infusion. The L4-L6 segments of the spinal cord were harvested right after the last behavioral test. The (A) PWT to von Frey filament stimulation and (B) PWL to thermal stimulation were evaluated 24 h before the incision and 2, 6, 24, and 48 h after remifentanil infusion. The data are presented as the mean ± SD (n = 8/group). (C) The p-NR1 and GLT-1 expression were measured by western blotting, and the quantitative analysis values of (D) p-NR1 and (E) GLT-1 are presented as the ratio of relative protein density to β-actin. The data are presented as the mean ± SD (n = 4/group). *P < 0.05 vs. Normal saline + Vehicle group, #P < 0.05 vs. Remifentanil + Vehicle group. IL-1ra, IL-1β inhibitor antagonist; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; p-NR1, phospho-NMDA receptor NR1; GLT-1, glutamate transporter-1.

published an article indicated that spinal NLRP3 inflammasome activation and associated release of IL-1β play an important role in opioid-induced hyperalgesia (OIH).[10] Inspired by this article, we decided to confirm if the NLRP3 inflammasome activation and its downstream axis could also play the key role in the postoperative RIH. In the present study, 1.2 μg/kg/min was adopted as the remifentanil infusion rate based on our previous studies.[7,16,24] The effect of A438079 appeared at 12.46 ± 3.98min after infusion and lasted for 1 week.[25] Ac-YVAD-cmk showed effect in 30min after infusion and lasted for 5 days.[26,27] The effect of IL-1ra appeared in 10 min after infusion and lasted 2 weeks or more.[28,29] (+)-naloxone appeared in 45min after infusion and lasted 4 days.[30,31] It is known that RIH in rats arises 2 h after remifentanil infusion and peaks 24–48 h after it.[32] RIH lasts for at least 7 days after remifentanil infusion.[33] Therefore, 24 h before surgery, and 2, 6, 24 and 48 h after remifentanil infusion were selected as the time points for behavioral tests. And the L4-L6 segments of the spinal cord were collected after the last behavioral test (48 h after remifentanil infusion).

Several reported studies have suggested that neuroimmune signaling accompanies OIH. Carranza-Aguilar et al.[34] reported that incisions and fentanyl administration increased the expression of IL-1β, IL-6, and TNF-α in the spinal cord. Hong et al.[35] reported that spinal astrocyte activation and inflammatory reaction are involved in RIH. Lv et al.[36] reported that the attenuation of RIH by betulinic acid was associated with a potential inhibition of oxidative stress and subsequent downregulation of IL-1β in the spinal dorsal horn. The current study showed that intraoperative infusion of remifentanil downregulated the nociceptive thresholds (PWT and PWL) at different time points (2, 6, 24, and 48 h) during the postoperative period (Figure 2). Moreover, remifentanil infusion could also enhance the mRNA and protein levels of IL-1β, increase phospho-NR1 and decrease GLT-1 expression. However, both the remifentanil-induced behavioral (mechanical and thermal hyperalgesia) and molecular biological (increased phospho-NR1 and decreased GLT-1 expression) changes could be reversed by pretreatment with IL-1ra a selective inhibitor of IL-1β.

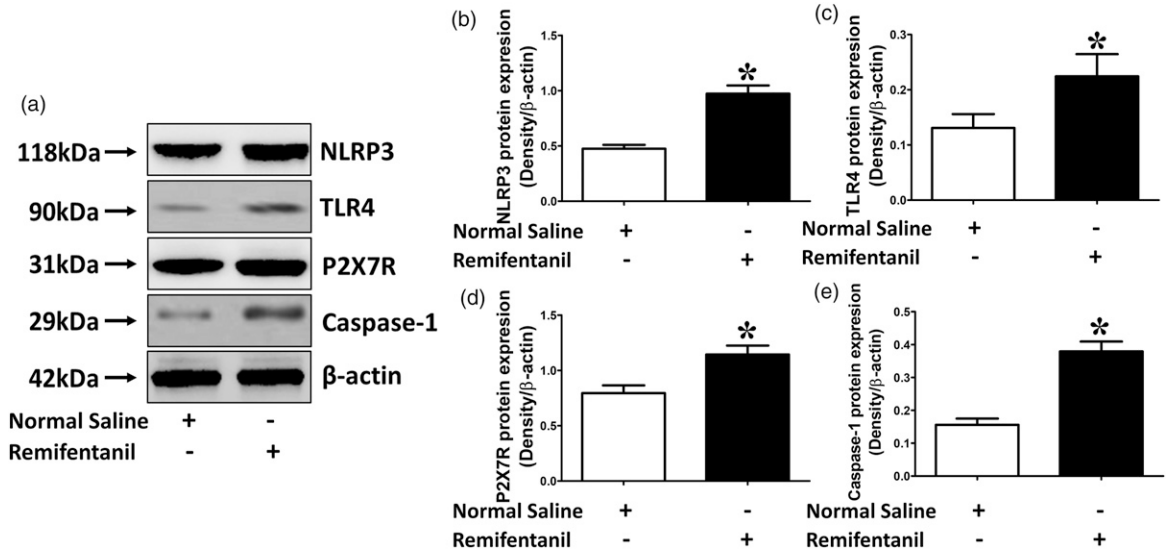

Figure 5. Expression of proteins associated with NLRP3 inflammasome activation after remifentanil infusion in rats. The L4-L6 segments of the spinal cord were harvested right after the last behavioral test. (A) The NLRP3, TLR4, P2X7R, and caspase-1 expression were measured by western blotting, and the quantitative analysis values of (B) NLRP3, (C) TLR4, (D) P2X7R, and (E) caspase-1 are presented as the ratio of relative protein density to β-actin. The data are presented as the mean ± SD (n = 4/group). *P < 0.05 vs. Normal saline group. NLRP3, NOD-like receptor protein 3; TLR4, Toll-like receptor 4; P2X7R, P2X purinoceptor 7.

It was indicated that (i) the spinal pro-inflammatory cytokine IL-1β, which is a crucial mediator of the inflammatory cascade, was increased by an incision and further amplified by remifentanil; (ii) phospho-NR1 was elevated, whereas GLT-1 was decreased by the combination of an incision and remifentanil; (iii) the inhibition of IL-1β before remifentanil administration prevented hyperalgesia, and (iv) the inhibition of IL-1β before remifentanil administration reversed the changes in phosphor-NR1 and GLT-1. These findings suggested that IL-1β contributes to RIH through the regulation of phospho-NR1 and GLT-1.

In addition, experiments were designed to test whether spinal NLRP3 inflammasome activation caused RIH. Thus, the NLRP3 inflammasome platform was pharmacologically inhibited at several levels, followed by an assessment of the behavioral and biochemical consequences of RIH. Spinal TLR4 was explored as the first sign of NLRP3 inflammasome activation. Intrathecal infusion of the TLR4 antagonist (+)-naloxone prevented the development of RIH.[37] Next, the role of spinal P2X7R was explored as the second sign of NLRP3 inflammasome activation. Intrathecal infusion of A438079, a selective P2X7R antagonist, prevented RIH development. The role of spinal caspase-1 was then explored, as it is the enzyme responsible for the proteolytic activation of IL-1β .[38] The intrathecal infusion of a caspase-1 antagonist, N-Ac-Tyr-Val-Ala-Asp-chloromethyl ketone (ac-YVAD-cmk),[39] prevented RIH development. The current study showed that the intraoperative infusion of remifentanil activated the NLRLP3 inflammasome by improving the expression of NLRP3, TLR4, P2X7R, and caspase-1 in the spinal cords of rats (Figure 5). More importantly, pretreatment with (+)-naloxone (an inhibitor of TLR4), A438079 (an inhibitor of P2X7R), and ac-YVAD-cmk (an inhibitor of caspase-1) could attenuate the development of remifentanil-induced mechanical and thermal hyperalgesia during the postoperative period, potentially through the regulation of IL-1β, phospho-NR1 and GLT-1 (Figure 6). These findings proved that RIH depends on TLR4, P2X7R, and caspase-1 signaling. It could therefore be concluded that spinal NLRP3 inflammasome activation caused RIH.

Phospho-NR1 and GLT-1 are markers of IL-1β-induced neuroexcitation. These were quantified following the administration of (+)-naloxone, A438079, and ac-YVAD-cmk. All inhibitors decreased the expression of phospho-NR1 and increased that of GLT-1 relative to the vehicle controls. These findings provided biochemical support for the attenuated IL-1β signaling. In the present study, spinal NLRP3 inflammasome activation was implicated in RIH. It was found that spinal NLRP3 inflammasomes mediate the initiation of RIH because i) NLRP3, TLR4, P2X7R, and caspase-1 were elevated by remifentanil infusion and ii) the inhibition of TLR4, P2X7R, or caspase-1 before remifentanil administration prevented hyperalgesia. The mechanisms through which remifentanil activated NLRP3 inflammasomes are unknown. The present results suggested that the expression of IL-1β was increased by an incision and further amplified by remifentanil. Then increase in IL-1β mediated the downregulation of GLT-1, followed by an increase in glutamate. The increase in glutamate may trigger ATP release from glial cells.[40] ATP has been shown to activate P2X7R, the second sign of NLRP3 inflammasome activation.[41,42] It has also been demonstrated that OIH induced by systemic low-dose morphine shares action at the

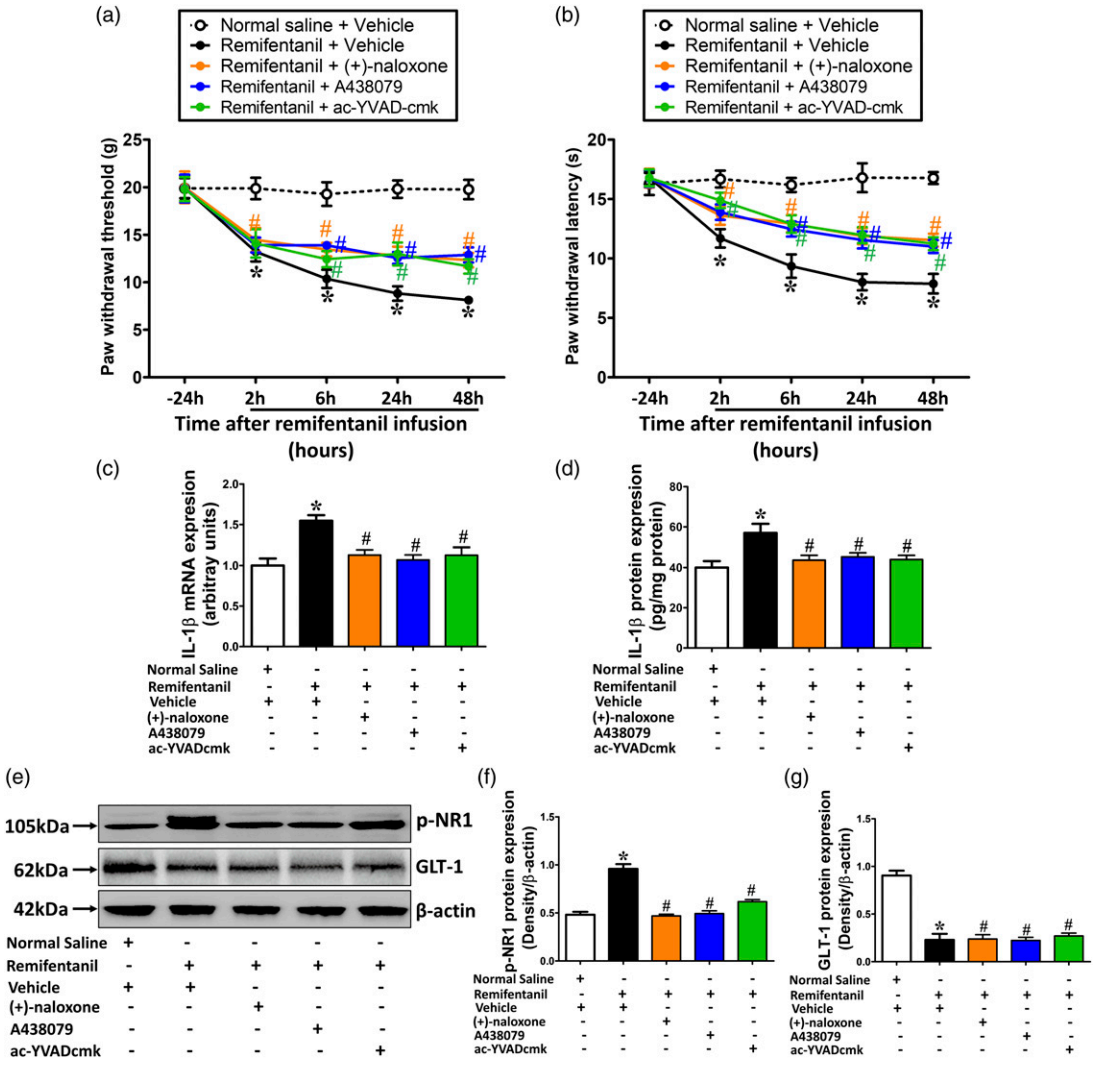

Figure 6. After remifentanil infusion, the effects of NLRP3 inflammasome activation inhibitors on behavioral changes, IL-1β, p-NR1, and GLT-1 expression in rats. Three different selective NLRP3 inflammasome activation inhibitors, (+)-Naloxone (a TLR4 inhibitor; 1,200 μg dissolved in 10 μl vehicle), A438079 (a P2X7R inhibitor; 600 ng dissolved in 10 μl vehicle), and ac-YVAD-cmk (a caspase-1 inhibitor; 20 μg dissolved in 10 μl vehicle) or vehicle (0.15 μl DMSO dissolved in 10 μl saline) was intrathecally administered into the rats respectively before remifentanil infusion. The L4-L6 segments of the spinal cord were harvested immediately after the last behavioral test. The (A) PWT to von Frey filament stimulation and (B) PWL to thermal stimulation were evaluated 24 h before the incision and 2, 6, 24, and 48 h after remifentanil infusion. The data are presented as the mean ± SD (n=8/group). (C) mRNA and (D) protein levels of IL-1β were detected by RT-qPCR and ELISA, respectively. (E) The p-NR1 and GLT-1 expression were measured by western blotting, and the quantitative analysis values of (F) p-NR1 and (G) GLT-1 are presented as the ratio of relative protein density to μ-actin. The data are presented as the mean ± SD (n=4/group). *P<0.05 vs. Normal saline + Vehicle group; #P<0.05 vs. Remifentanil + Vehicle group. NLRP3, NOD-like receptor protein 3; TLR4, Toll-like receptor 4; P2X7R, P2X purinoceptor 7; PWT, paw withdrawal threshold; PWL, paw withdrawal latency; p-NR1, phospho-NMDA receptor NR1; NMDA receptor NR1; GLT-1, glutamate transporter-1; RT-qPCR, reverse quantitative transcription PCR.

TLR4 protein and signaling via protein kinase Cε.[43] However, the potential mechanism underlying NLRP3 inflammasome activation is unclear and requires further research.

The present study demonstrated how NLRP3 inflammasome activation contributes to RIH. It was confirmed that the inhibition of TLR4, P2X7R, or caspase-1 before remifentanil administration reversed the increase in IL-1β and phospho-NR1 and the decrease in GLT-1, which was induced by incisions and remifentanil. Of note, the release of mature IL-1β requires the cleavage of the precursor protein pro-IL-1β. Pro-IL-1β separation is achieved through caspase-1, which is activated following the formation and activation of the NLRP3 inflammasome. This result demonstrated that NLRP3 inflammasome inhibition could prevent RIH by regulating the levels of IL-1β, phospho-NR1, and GLT-1.

However, the present study had several limitations. First, we only employed Remifentanil intravenously infusion to set the RIH model without surgery on the animals. Because

surgery-related trauma can lead to a systemic inflammatory response, which can result in multi-organ dysfunction or even failure.[44] For studying the mechanism of RIH more intuitively, we did not operate on animals. Second, only male rats were used in the study. Female rats have been proven to be sensitive to hormonal alterations.[45] As a result, male rats' physiological indications are more stable than female ones. However, diverse perspectives have suggested no substantial difference in specific functional results between male and female animals following injuries.[46] We'll try to use animals of different genders in our future research. Third, the main signaling pathway about effects of NLRP3 activation and IL-1β release on OIH has already be reported before.[10] However, in this research, we have expanded the scope of application of this pathway, and we found it also play an important role in RIH.

Conclusions

In conclusion, NLRP3 inflammasome activation contributes to RIH by regulating phospho-NR1 and GLT-1 by IL-1β. The inhibition of NLRP3 inflammasome activation may be an effective and novel strategy for treating RIH.

Authors' contributions

Yuan Yuan: Methodology, Writing-original draft
Yue Zhao: Methodology and Data curation
Mengxi Shen: Methodology, Software, Data curation
Chenxu Wang: Software, Conceptualization
Beibei Dong: Methodology, Software
Keliang Xie: Methodology
Yang Yu: Data curation, Writing-review and editing
Yonghao Yu: Supervision.

Declaration of Conflicting Interests

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References

1. Moy JK, Hartung JE, Duque MG. Distribution of functional opioid receptors in human dorsal root ganglion neurons. *Pain* 2020; 161: 1636–1649. DOI: 10.1097/j.pain.0000000000001846
2. Yi P, Pryzbylkowski P. Opioid Induced Hyperalgesia. *Pain Med* 2015; 16(Suppl 1): S32–S36. DOI: 10.1111/pme.12914
3. Shu RC, Zhang LL, Wang CY. Spinal peroxynitrite contributes to remifentanil-induced postoperative hyperalgesia via enhancement of divalent metal transporter 1 without iron-responsive element-mediated iron accumulation in rats. *Anesthesiology* 2015; 122: 908–920. DOI: 10.1097/ALN.0000000000000562
4. Qi F, Liu T, Zhang X. Ketamine reduces remifentanil-induced postoperative hyperalgesia mediated by CaMKII-NMDAR in the primary somatosensory cerebral cortex region in mice. *Neuropharmacology* 2020; 162: 107783. DOI: 10.1016/j.neuropharm.2019.107783
5. Hu J, Chen S, Zhu M. Preemptive Nalbuphine Attenuates Remifentanil-Induced Postoperative Hyperalgesia After Laparoscopic Cholecystectomy: A Prospective Randomized Double-Blind Clinical Trial. *J Pain Res* 2020; 13: 1915–1924. DOI: 10.2147/JPR.S257018
6. Kim SH, Stoicea N, Soghomonyan S. Intraoperative use of remifentanil and opioid induced hyperalgesia/acute opioid tolerance: systematic review. *Front Pharmacol* 2014; 5: 108. DOI: 10.3389/fphar.2014.00108
7. Wang C, Li Q, Jia Z. Spinal caspase-6 contributes to remifentanil-induced hyperalgesia via regulating CCL21/CXCR3 pathway in rats. *Neurosci Lett* 2020; 721: 134802. DOI: 10.1016/j.neulet.2020.134802
8. Yuan Y, Wang JY, Yuan F. Glycogen synthase kinase-3beta contributes to remifentanil-induced postoperative hyperalgesia via regulating N-methyl-D-aspartate receptor trafficking. *Anesth Analg* 2013; 116: 473–481. DOI: 10.1213/ANE.0b013e318274e3f1
9. Wang Z, Yuan Y, Xie K. PICK1 Regulates the Expression and Trafficking of AMPA Receptors in Remifentanil-Induced Hyperalgesia. *Anesth Analg* 2016; 123: 771–781. DOI: 10.1213/ANE.0000000000001442
10. Grace PM, Strand KA, Galer EL. Morphine paradoxically prolongs neuropathic pain in rats by amplifying spinal NLRP3 inflammasome activation. *Proc Natl Acad Sci U S A* 2016; 113: E3441–E3450. DOI: 10.1073/pnas.1602070113
11. Grace PM, Galer EL, Strand KA. Repeated Morphine Prolongs Postoperative Pain in Male Rats. *Anesth Analg* 2019; 128: 161–167. DOI: 10.1213/ANE.0000000000003345
12. Grace PM, Strand KA, Galer EL. Protraction of neuropathic pain by morphine is mediated by spinal damage associated molecular patterns (DAMPs) in male rats. *Brain Behav Immun* 2018; 72: 45–50. DOI: 10.1016/j.bbi.2017.08.018
13. Green-Fulgham SM, Ball JB, Maier S. Suppression of active phase voluntary wheel running in male rats by unilateral chronic constriction injury: Enduring therapeutic effects of a brief treatment of morphine combined with TLR4 or P2X7 antagonists. *J Neurosci Res* 2022; 100: 265–277. DOI: 10.1002/jnr.24645
14. Zhang RX, Li A, Liu B. IL-1ra alleviates inflammatory hyperalgesia through preventing phosphorylation of NMDA receptor NR-1 subunit in rats. *Pain* 2008; 135: 232–239. DOI: 10.1016/j.pain.2007.05.023
15. Yan X, Yadav R, Gao M. Interleukin-1 beta enhances endocytosis of glial glutamate transporters in the spinal dorsal horn

through activating protein kinase C. *Glia* 2014; 62: 1093–1109. DOI: 10.1002/glia.22665

16. Yuan Y, Sun Z, Chen Y. Prevention of Remifentanil Induced Postoperative Hyperalgesia by Dexmedetomidine via Regulating the Trafficking and Function of Spinal NMDA Receptors as well as PKC and CaMKII Level In Vivo and In Vitro. *PLoS One* 2017; 12: e0171348. DOI: 10.1371/journal.pone.0171348
17. Vasefi MS, Yang K, Li J. Acute 5-HT7 receptor activation increases NMDA-evoked currents and differentially alters NMDA receptor subunit phosphorylation and trafficking in hippocampal neurons. *Mol Brain* 2013; 6: 24. DOI: 10.1186/1756-6606-6-24
18. Qu X, Xu C, Wang H. Hippocampal glutamate level and glutamate aspartate transporter (GLAST) are up-regulated in senior rat associated with isoflurane-induced spatial learning/memory impairment. *Neurochem Res* 2013; 38: 59–73. DOI: 10.1007/s11064-012-0889-8
19. Pajarillo E, Rizor A, Lee J. The role of astrocytic glutamate transporters GLT-1 and GLAST in neurological disorders: Potential targets for neurotherapeutics. *Neuropharmacology* 2019; 161: 107559. DOI: 10.1016/j.neuropharm.2019.03.002
20. Kilkenny C, Browne WJ, Cuthill IC. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *Plos Biol* 2010; 8: e1000412. DOI: 10.1371/journal.pbio.1000412
21. Milligan ED, Hinde JL, Mehmert KK. A method for increasing the viability of the external portion of lumbar catheters placed in the spinal subarachnoid space of rats. *J Neurosci Methods* 1999; 90: 81–86. DOI: 10.1016/s0165-0270(99)00075-8
22. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008; 3: 1101–1108. DOI: 10.1038/nprot.2008.73
23. Zhao Q, Zhang L, Shu R. Involvement of Spinal PKMzeta Expression and Phosphorylation in Remifentanil-Induced Long-Term Hyperalgesia in Rats. *Cell Mol Neurobiol* 2016; 37(4): 643–653. DOI: 10.1007/s10571-016-0401-0
24. Wang C, Xu R, Wang X. Spinal CCL1/CCR8 regulates phosphorylation of GluA1-containing AMPA receptor in postoperative pain after tibial fracture and orthopedic surgery in mice. *Neurosci Res* 2019; 150: 20–26. DOI: 10.1016/j.neures.2019.05.003
25. Kim JE, Kang TC. The P2X7 receptor-pannexin-1 complex decreases muscarinic acetylcholine receptor-mediated seizure susceptibility in mice. *J Clin Invest* 2011; 121: 2037–2047. DOI: 10.1172/JCI44818
26. Song P, Yi Z, Fu Y. Reversing Postcardiopulmonary Bypass Associated Cognitive Dysfunction Using k-Opioid Receptor Agonists to Regulate Microglial Polarization via the NLRP3/Caspase-1 Pathway. *J Healthc Eng* 2021; 2021: 3048383–3052021. DOI: 10.1155/2021/3048383
27. Qu W, Wang Y, Wu Y. Triggering Receptors Expressed on Myeloid Cells 2 Promotes Corneal Resistance Against Pseudomonas aeruginosa by Inhibiting Caspase-1-Dependent Pyroptosis. *Front Immunol* 2018; 9.
28. Wang F, Liu J, Chen X. IL-1beta receptor antagonist (IL-1Ra) combined with autophagy inducer (TAT-Beclin1) is an effective alternative for attenuating extracellular matrix degradation in rat and human osteoarthritis chondrocytes. *Arthritis Res Ther* 2019; 21.
29. Jin C, Fu WL, Zhang DD. The protective role of IL-1Ra on intestinal ischemia reperfusion injury by anti-oxidative stress via Nrf2/HO-1 pathway in rat. *Biomed J* 2019; 42: 36–45. DOI: 10.1016/j.bj.2018.11.001
30. Li X, Wang XW, Feng XM. Stage-dependent anti-allodynic effects of intrathecal Toll-like receptor 4 antagonists in a rat model of cancer induced bone pain. *J Physiol Sci* 2013; 63: 203–209. DOI: 10.1007/s12576-012-0244-5
31. Ruiz-Perez D, Benito J, Polo G. The Effects of the Toll-Like Receptor 4 Antagonist, Ibudilast, on Sevoflurane's Minimum Alveolar Concentration and the Delayed Remifentanil-Induced Increase in the Minimum Alveolar Concentration in Rats. *Anesth Analg* 2016; 122: 1370–1376. DOI: 10.1213/ANE.0000000000001171
32. Gu X, Wu X, Liu Y. Tyrosine phosphorylation of the N-Methyl-D-Aspartate receptor 2B subunit in spinal cord contributes to remifentanil-induced postoperative hyperalgesia: the preventive effect of ketamine. *Mol Pain* 2009; 5: 76. DOI: 10.1186/1744-8069-5-76
33. Cabanero D, Campillo A, Celerier E. Pronociceptive effects of remifentanil in a mouse model of postsurgical pain: effect of a second surgery. *Anesthesiology* 2009; 111: 1334–1345. DOI: 10.1097/ALN.0b013e3181bfab61
34. Carranza-Aguilar CJ, Hernandez-Mendoza A, Mejias-Aponte C. Morphine and Fentanyl Repeated Administration Induces Different Levels of NLRP3-Dependent Pyroptosis in the Dorsal Raphe Nucleus of Male Rats via Cell-Specific Activation of TLR4 and Opioid Receptors. *Cel Mol Neurobiol* 2020; 42(3): 677–694. DOI: 10.1007/s10571-020-00957-5
35. Hong HK, Ma Y, Xie H. TRPV1 and spinal astrocyte activation contribute to remifentanil-induced hyperalgesia in rats. *Neuroreport* 2019; 30: 1095–1101. DOI: 10.1097/WNR.0000000000001329
36. Lv CC, Xia ML, Shu SJ. Attenuation of Remifentanil-Induced Hyperalgesia by Betulinic Acid Associates with Inhibiting Oxidative Stress and Inflammation in Spinal Dorsal Horn. *Pharmacology* 2018; 102: 300–306. DOI: 10.1159/000493144
37. Hutchinson MR, Zhang Y, Brown K. Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). *Eur J Neurosci* 2008; 28: 20–29. DOI: 10.1111/j.1460-9568.2008.06321.x
38. de Rivero Vaccari JP, Dietrich WD, Keane RW. Activation and regulation of cellular inflammasomes: gaps in our knowledge for central nervous system injury. *J Cereb Blood Flow Metab* 2014; 34: 369–375. DOI: 10.1038/jcbfm.2013.227
39. Rabuffetti M, Sciorati C, Tarozzo G. Inhibition of caspase-1-like activity by Ac-Tyr-Val-Ala-Asp-chloromethyl ketone induces long-lasting neuroprotection in cerebral ischemia through apoptosis reduction and decrease of proinflammatory cytokines. *J Neurosci* 2000; 20: 4398–4404.
40. Liu GJ, Kalous A, Werry EL. Purine release from spinal cord microglia after elevation of calcium by glutamate. *Mol Pharmacol* 2006; 70: 851–859. DOI: 10.1124/mol.105.021436

41. Zhao H, Kilgas S, Alam A. The role of extracellular adenosine triphosphate in ischemic organ injury. *Crit Care Med* 2016; 44: 1000–1012. DOI: 10.1097/CCM.0000000000001603
42. Ficker C, Rozmer K, Kato E. Astrocyte-neuron interaction in the substantia gelatinosa of the spinal cord dorsal horn via P2X7 receptor-mediated release of glutamate and reactive oxygen species. *Glia* 2014; 62: 1671–1686. DOI: 10.1002/glia.22707
43. Araldi D, Bogen O, Green PG. Role of Nociceptor Toll-like Receptor 4 (TLR4) in Opioid-Induced Hyperalgesia and Hyperalgesic Priming. *J Neurosci* 2019; 39: 6414–6424. DOI: 10.1523/JNEUROSCI.0966-19.2019
44. Alam A, Hana Z, Jin Z. Surgery, neuroinflammation and cognitive impairment. *EBioMedicine* 2018; 37: 547–556. DOI: 10.1016/j.ebiom.2018.10.021
45. Iqbal J, Adu-Nti F, Wang X. Sex difference in depression: Which animal models mimic it. *Behav Neurosci* 2020; 134: 248–266. DOI: 10.1037/bne0000369.
46. Limatola V, Ward P, Cattano D. Xenon preconditioning confers neuroprotection regardless of gender in a mouse model of transient middle cerebral artery occlusion. *Neuroscience* 2010; 165: 874–881. DOI: 10.1016/j.neuroscience.2009.10.063