Animal Study

Lipocalin-2 in the Anterior Cingulate Cortex Contributes to Remifentanil-Induced Postoperative Hyperalgesia

Jing Shen, MD[1], Yilong Wang, MD[1], Weijie Xie, MD, PhD[2], Qing Luo, MD[1], Fanhua Meng, MD[1], Henry Liu, MD[3], and Zeyong Yang, MD, PhD[1]

From: [1]Department of Anesthesiology, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Embryo Original Disease, Shanghai Municipal Key Clinical Specialty; Shanghai Jiao Tong University School of Medicine, Shanghai, China; [2]Clinical Research Center for Mental Disorders, Shanghai Pudong New Area Mental Health Center, Tongji University School of Medicine, Shanghai, China; [3]Department of Anesthesiology and Critical Care, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Address Correspondence:
Zeyong Yang, MD, PhD
Department of Anesthesiology, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Embryo Original Disease, Shanghai Municipal Key Clinical Specialty, Shanghai Jiaotong University School of Medicine
No. 910, Hengshan Road
Xuhui District
Shanghai, China, 200030
E-mail: yankylge@aliyun.com

Disclaimer: This work was supported by the Medical and Engineering Crossover Key Project of Shanghai Jiao Tong University (No: YG2022ZD029).

Conflict of interest: Each author certifies that he or she, or a member of his or her immediate family, has no commercial association (i.e., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted article.

Article received: 07-03-2024
Revised article received: 07-19-2024
Accepted for publication: 09-09-2024

Free full article: www.painphysicianjournal.com

Background: Opioids, which are widely used during surgery in perioperative settings, may cause hyperalgesia, especially when the opioid employed is remifentanil. Opioid-induced hyperalgesia may increase the length of a patient's hospital stay and negatively affect enhanced recovery after surgery and the patient's prognosis. Currently, there is no consensus on treatment strategies for remifentanil-induced postoperative hyperalgesia (RIPH).

Objectives: This study aimed to test the hypothesis that upregulation of lipocalin-2 (LCN2) in the anterior cingulate cortex (ACC) contributes to RIPH.

Study Design: A controlled animal study.

Setting: A university laboratory.

Methods: The RIPH mouse model was established through the subcutaneous infusion of remifentanil in mice undergoing plantar incision surgery. The von Frey test and the Hargreaves test were used to measure the pain threshold. By combining RNA sequencing, Western blotting, in vivo pharmacology, and the construction of adeno-associated virus vectors that modulated the expression level of LCN2 specifically, the role of LCN2 in the occurrence of RIPH in mice was explored.

Results: Compared to the mice that were subjected to the combination of incisions and saline (inci + saline), mice subjected to incisions and remifentanil (inci + remi) did not experience a significant reduction in the mechanical pain threshold of their ipsilateral hind paws. The mechanical pain threshold of the contralateral hind paws of the inci + remi mice was significantly reduced compared to those of the inci + saline mice. According to transcriptome analysis, LCN2 expression was significantly upregulated in RIPH-model mice. Furthermore, the Western blotting analysis also showed a significant increase in the level of LCN2 in the ipsilateral ACC of RIPH-model mice. An intra-anterior cingulate cortex injection of LCN2 mAb could attenuate hyperalgesia in mice. Knockdown of LCN2 expression in the ACC significantly alleviated mechanical hyperalgesia in mice. Additionally, the overexpression of LCN2 in the ACC could directly induce mechanical hyperalgesia without affecting thermal nociception.

Limitations: Future research needs to explore more potential mechanisms of affecting pain sensitivity through LCN2 upregulation.

Conclusions: Our results demonstrated that the upregulation of LCN2 in the ACC plays a crucial role in the occurrence of RIPH, suggesting that LCN2 potentially be a therapeutic target for alleviating RIPH.

Key words: Remifentanil, Lipocalin-2, hyperalgesia, postoperative hyperalgesia, anterior cingulate cortex, opioid-induced hyperalgesia, enhanced recovery after surgery, opioid.

Pain Physician 2025: 28:E49-E59

Postoperative opioid-induced hyperalgesia (OIH) is a common complication that follows the administration of opioids. OIH may seriously affect patients' recovery and quality of life (1). Numerous investigations have shown that the increase in early postoperative pain sensitivity may be associated with the potential ability of intraoperatively administered analgesics, especially opioids, to control surgical pain and nociceptive effects of surgery (2). OIH is a contradictory and concerning phenomenon, especially when it is associated with the use of short-acting opioids such as remifentanil (3, 4). Remifentanil is a μ-opioid receptor agonist used widely for intraoperative antinociception and analgesia (5,6). Due to its unique pharmacokinetics with rapid onset and offset, remifentanil can be safely used at higher doses without delaying postoperative recovery (7,8). However, clinical studies have found that high doses of remifentanil during surgery can significantly increase the severity of acute pain after surgery (3). In addition, animal models have clearly demonstrated remifentanil-induced postoperative hyperalgesia (RIPH) (9).

Pain is a subjective experience induced by the complex interactions among afferent sensory inputs and their processing neurons throughout the nervous system, from the periphery to the spinal cord and the brain (10). Previous animal studies on OIH, which have focused primarily on the spinal cord, have found that the activation of the spinal glial is necessary for central sensitization in OIH (11). In recent years, studies have discovered that cortical and thalamic regions such as the anterior cingulate cortex (ACC), hippocampus, primary somatosensory cortex, and ventral posterolateral nucleus are all related to the development of OIH (9,12,13). Human brain imaging studies have confirmed that the ACC can be activated by noxious stimuli (14,15). A functional magnetic resonance imaging (fMRI) study conducted on healthy volunteers found that the short-term infusion of remifentanil followed by drug withdrawal increased functional connectivity between the nucleus cuneiformis (NCF) and the rostral anterior cingulate cortex (rACC). The strength of the rACC-NCF connectivity was negatively correlated with the individual heat pain threshold after applying remifentanil. These findings suggest that ACC plays a crucial role in human pain processing (16). Studies in rodents have demonstrated that increased ACC neuronal activity is involved in developing neuropathic pain and chronic restraint stress-induced hyperalgesia in mice (17,18). These studies suggested that increased neuronal activity in the ACC is closely related to the occurrence and development of acute and chronic pain. However, it is still unclear what cellular and molecular mechanisms within the cortical network contribute to the pathogenesis of RIPH.

Lipocalin-2, known as a neutrophil gelatinase-associated lipocalin (NGAL) or 24p3, is an acute-phase protein involved in various physiological and pathological processes (19). In a chronic inflammatory pain animal model induced by complete Freund's adjuvant (CFA), LCN2 was strongly induced in the ipsilateral hind limb injected with CFA (20). In the central nervous system, LCN2 can be produced and secreted by neurons, microglia, and astrocytes and regulates neural excitability (21-23). Some studies have found that LCN2 mediates neuropathic pain by inducing chemokine expression and subsequent microglial activation (24).

This study aimed to investigate the role of LCN2 in RIPH. We hypothesised that hind-paw incisions and remifentanil administrations in mice would induce the overexpression of LCN2 in the ACC, leading to hyperalgesia. As the enhanced recovery after surgery (ERAS) procedure and the same-day discharge (SDD) program have risen in popularity, the role of remifentanil in these processes has become increasingly concerning (25,26). The present study is intended to increase our understanding of the mechanism underlying the development of OIH.

Methods

Animals

Male C57BL/6J mice 8-10 weeks of age were purchased from Charles River Laboratories International, Inc. The mice were housed in a specific pathogen-free (SPF) facility with a cycle of 12 hours of light and 12 hours of darkness. They had free access to food and water. All animal studies were performed in strict accordance with the Guide of the National Institutes of Health (NIH) for the Care and Use of Laboratory Animals and approved by the Medical Research Ethics Committee of International Peace Maternity and Child Health Hospital (GKLW 2021-32).

Plantar Incision Surgery

Mice were anesthetized with 2% to 3% isoflurane via a nose cone in a sterile operating room. A longitudinal incision of 7 mm was made on each mouse's left plantar, starting 2 mm from the proximal edge of the heel and extending toward the toes through the skin

and fascia. The muscle was lifted with curved forceps and longitudinally incised while preserving its origin and insertion (27). The skin was sutured using a 6-0 nylon thread. The wound was treated with erythromycin ointment to prevent infection.

Drugs

Remifentanil was purchased from Yichang Renfu Pharmaceutical Co., Ltd., and isoflurane was obtained from RWD Life Science Co., Ltd. Remifentanil (40 μg/kg) (9) was dissolved in saline (0.9% NaCl) solution and infused subcutaneously at a rate of 1.2 mL/h in 30 minutes using a Harvard Apparatus pump. The mice in the control group were administered an equal volume of saline solution under the same conditions.

Pain Behavior Tests

Von Frey filament stimuli quantified mechanical hyperalgesia. Mice were habituated to an experiment-handling technician and behavioral chamber for 3 consecutive days before the experiments. The mice were placed in custom-made polymethyl methacrylate cubicles (8x8x10 cm) on a perforated metal floor and were habituated for at least one hour before testing. The Dixon up-down method was used to estimate 50% withdrawal thresholds, using calibrated von Frey nylon monofilaments (Stoelting Co.). Filaments were applied to the plantar surface of the mice's hind paws for 3 seconds, and responses were recorded. A nociceptive-like response was considered when paw withdrawal or licking was observed. On each mouse, both hind paws were tested.

The Hargreaves test was used to assess the thermal nociceptive threshold. For 3 consecutive days before the experiment, each mouse was placed in a standard polymethyl methacrylate enclosure on a glass platform for 30 minutes daily to acclimatize. An emission of radiant heat (IITC Life Science, Inc.) under the glass was focused perpendicularly on the midplantar surface. Paw withdrawal latency was defined as the time (in seconds) from the start of heat exposure until hind paw withdrawal. If no response was observed at a latency of 20 seconds, the test was stopped to avoid tissue damage.

Virus Injection

Mice were anesthetized, maintained with isoflurane, and placed on a stereotaxic apparatus (RWD Life Science Co., Ltd.). During the surgery, erythromycin eye ointment was applied to keep the mice's eyes lubricated. To knockdown the expression of LCN2, AAV-U6-shRNA (LCN2)-CMV-EGFP was injected into the left ACC (virus titers: 5-6×10^{12} vg/mL, 400 nL/inject, AP, +0.90 mm; ML, +0.28 mm; DV, –1.50 mm). AAV-U6-shRNA (scramble)-CMV-EGFP was used as a control (virus titers: 5-6×10^{12} vg/mL, 400 nL/inject, AP, +0.90 mm; ML, +0.28 mm; DV, –1.50 mm).

For overexpression of LCN2, AAV-CMV-LCN2-EGFP was injected into the left ACC (virus titers: 5-6 ×10^{12} vg/mL, 400 nL/inject, AP, +0.90 mm; ML, +0.28 mm; DV, –1.50 mm). AAV-CMV-EGFP was used as a control (virus titers: 5-6 ×10^{12} vg/mL, 400 nL/inject, AP, +0.90 mm; ML, +0.28 mm; DV, –1.50 mm).

The virus was injected at a rate of 50 nL/min. The glass microelectrode was left at the injection position for 10 minutes to allow the virus to spread after the injection and then move slowly out of the glass electrode. All of the above viruses were purchased from Brain VTA (Hubei, China).

In Vivo Pharmacological Methods

An anesthetized mouse was immobilized in a stereotactic frame, and a cannula (RWD Life Sciences Co., Ltd.) was implanted into its left ACC, supported by 2 skull-penetrating M1 screws and a dental acrylic. Either LCN2 mAb (0.65 mg/300 nL; # MAB1857, R&D System) (28) or isotype mAb (0.65 mg/300 nL; #MAB006, R&D System) was injected into the left ACC at a rate of 100 nL/min through the cannula 30 minutes before pain testing.

Western Blotting (WB)

The left ACC samples collected from the test mice under isoflurane anesthesia were processed for protein extraction using a RIPA lysis buffer supplemented with protease inhibitors. After centrifugation at 12,000 rpm for 15 minutes, the supernatants were collected, and the protein concentration was determined using a BCA Protein Assay Kit (Thermo Scientific™). Subsequently, the proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% (w/v) skim milk for 1.5 hours at room temperature and then incubated overnight at 4°C with antibodies against LCN2 (1:1000; #26991-1-AP, Proteintech) and β-actin (1:5000; #SB-AB0035, Share-Bio). The membranes were incubated with secondary antibodies (1:5000; #SA00001-2, Proteintech) for 1.5 hours at room temperature, and protein bands were detected using the ChemiDoc™ MP Imaging System

(Biorad). Finally, the protein levels were quantified using ImageJ software.

RNA Sequencing (RNA-Seq) and Data Analysis

Total RNA was extracted using the mirVana™ miRNA Isolation Kit (Ambion), following the manufacturer's protocol. RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies). The samples with RNA Integrity Number (RIN) ≥ 7 were subjected to subsequent analysis. The libraries were constructed using the TruSeq Stranded mRNA LTSample Prep Kit (Illumina) according to the manufacturer's instructions. These libraries were then sequenced on the Illumina sequencing platform (HiSeqTM 2500 or Illumina HiSeq X Ten), and 125bp/150bp paired-end reads were generated. The transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China). The DESeq2 software was utilized for analyzing differentially expressed genes (DEGs) between groups, with a threshold of $|\log2FC| > 2$ and P-value < 0.05.

Statistical Analysis

All data were calculated using GraphPad Prism 8 software and presented as mean ± standard error of mean (mean ± SEM). Statistical methods included 2-tailed unpaired t-test and two-way analysis of variance (ANOVA) test. $P < 0.05$, $P < 0.01$ and $P < 0.001$ were accepted as statistically significant. Data analysis was conducted by biostatisticians blinded to the experimental conditions.

Results

Remifentanil-Induced Postoperative Mechanical Hyperalgesia in Mice

We established a mouse model of RIPH by administering remifentanil (40 μg/kg) via subcutaneous injection for 30 minutes in mice that had undergone a left (ipsilateral) plantar incision (Fig. 1A). Compared with the baseline, plantar incision surgery can lower the mechanical pain threshold. However, compared to incised mice treated with saline (inci+saline), mice that received remifentanil (inci+remi) did not experience significant reductions in the mechanical pain threshold of their ipsilateral hind paws, according to the postoperative von Frey tests (Fig. 1C). This result might have been due to the hypersensitivity caused by incisional trauma, which makes it difficult to distinguish the degree of pain. Interestingly, von Frey tests showed that the mechanical pain threshold of the right hind paws (contralateral) of incised mice treated with remifentanil was significantly lower than those treated with saline (Fig. 1B). Concurrently, we observed no differences in the thermal pain threshold between the ipsilateral and contralateral hind paws of the 2 groups of mice in the Hargreaves tests (Figs. 1D, 1E). Therefore, in subsequent experiments, we focused on studying RIPH in the contralateral hind paws.

The Expression of LCN2 Is Upregulated in the ACC of RIPH Mice

As is widely known, nociceptive information generated in the periphery is sent to the ipsilateral spinal cord and then transmitted in a contralateral crossover manner, ultimately reaching relevant regions of the cerebral cortex for further processing and recognition (29). To gain a deeper understanding of the potential molecular mechanisms underlying abnormal neuronal structure and function in the ipsilateral ACC of mice with RIPH, we performed RNA-seq analysis to capture transcriptome-wide changes. The volcano plot visualizes the distribution of differentially expressed genes (DEGs) in the ipsilateral ACC, including 47 upregulated and 102 downregulated genes (Fig. 2A). Previous studies have confirmed that LCN2 regulates various biological and behavioral responses, including emotional behavior, depression, neuronal excitability, and anxiety (30). Through the analysis of DEGs, we found a significant increase in the expression level of LCN2 in the ipsilateral ACC of RIPH mice (Fig. 2B). Furthermore, the WB analysis showed that the expression level of LCN2 was significantly higher in the ipsilateral ACC of RIPH mice than in inci + saline mice (Figs. 2C, 2D). The ACC is a brain region closely related to emotional processing, decision-making, and pain perception. Therefore, changes in the expression of LCN2 in this region may directly affect these functions.

Intra-ACC Injection of LCN2 mAb Attenuated RIPH

To investigate whether the increased expression of LCN2 in the ACC affected pain sensitivity in RIPH mice, we implanted a cannula to administer a neutralizing antibody targeting LCN2 (LCN2 mAb) in the ipsilateral ACC of inci+remi mice (Figs. 3A, 3B). The results showed that after LCN2 mAb administration, the mechanical nociceptive pain threshold demonstrated significantly greater recovery in inci+remi mice than in inci+remi mice treated with isotype mAb on the first day after surgery (Fig. 3C). The results above indicated that re-

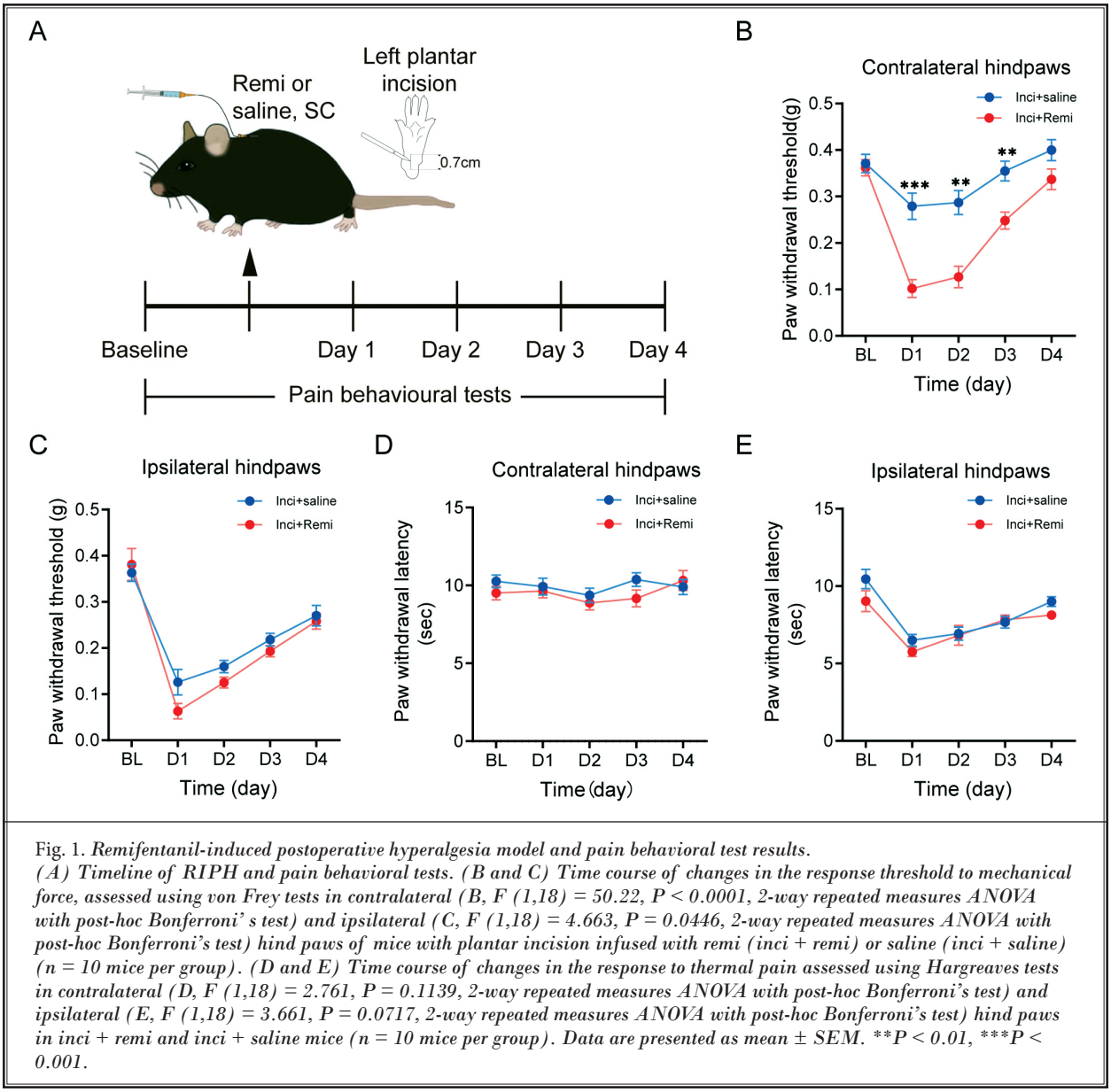

Fig. 1. *Remifentanil-induced postoperative hyperalgesia model and pain behavioral test results.*
*(A) Timeline of RIPH and pain behavioral tests. (B and C) Time course of changes in the response threshold to mechanical force, assessed using von Frey tests in contralateral (B, F (1,18) = 50.22, P < 0.0001, 2-way repeated measures ANOVA with post-hoc Bonferroni' s test) and ipsilateral (C, F (1,18) = 4.663, P = 0.0446, 2-way repeated measures ANOVA with post-hoc Bonferroni's test) hind paws of mice with plantar incision infused with remi (inci + remi) or saline (inci + saline) (n = 10 mice per group). (D and E) Time course of changes in the response to thermal pain assessed using Hargreaves tests in contralateral (D, F (1,18) = 2.761, P = 0.1139, 2-way repeated measures ANOVA with post-hoc Bonferroni's test) and ipsilateral (E, F (1,18) = 3.661, P = 0.0717, 2-way repeated measures ANOVA with post-hoc Bonferroni's test) hind paws in inci + remi and inci + saline mice (n = 10 mice per group). Data are presented as mean ± SEM. **P < 0.01, ***P < 0.001.*

ducing LCN2 levels in the ACC could alleviate mechanical hyperalgesia in RIPH mice.

LCN2 Knockdown Effectively Prevented RIPH

To explore whether the change in LCN2 within the ACC was involved in the remifentanil-induced development of mechanical hyperalgesia, taking into account the potential limitations of directly injecting neutralizing antibody into the brain region, we had also constructed/generated a broad-spectrum virus expressing short hairpin RNAs (shRNAs) to knockdown LCN2 expression. Three weeks after injecting AAV-U6-shRNA (LCN2)-CMV-EGFP (shLCN2) or AAV-U6-shRNA (scramble)-CMV-EGFP (scramble) into the ACC, we proceeded with the construction of the RIPH model (Figs. 4A, 4B). The WB analysis confirmed that LCN2 protein levels were significantly lower in LCN2 knockdown mice than in mice infected with the AAV-scramble control in the RIPH condition (Figs. 4C, 4D). The testing demonstrated that the inci+remi mice that had received ACC injections of AAV-shLCN2 showed significantly higher mechanical nociceptive pain thresholds than those injected with the AAV-scramble control virus, indicating that knocking down LCN2 in

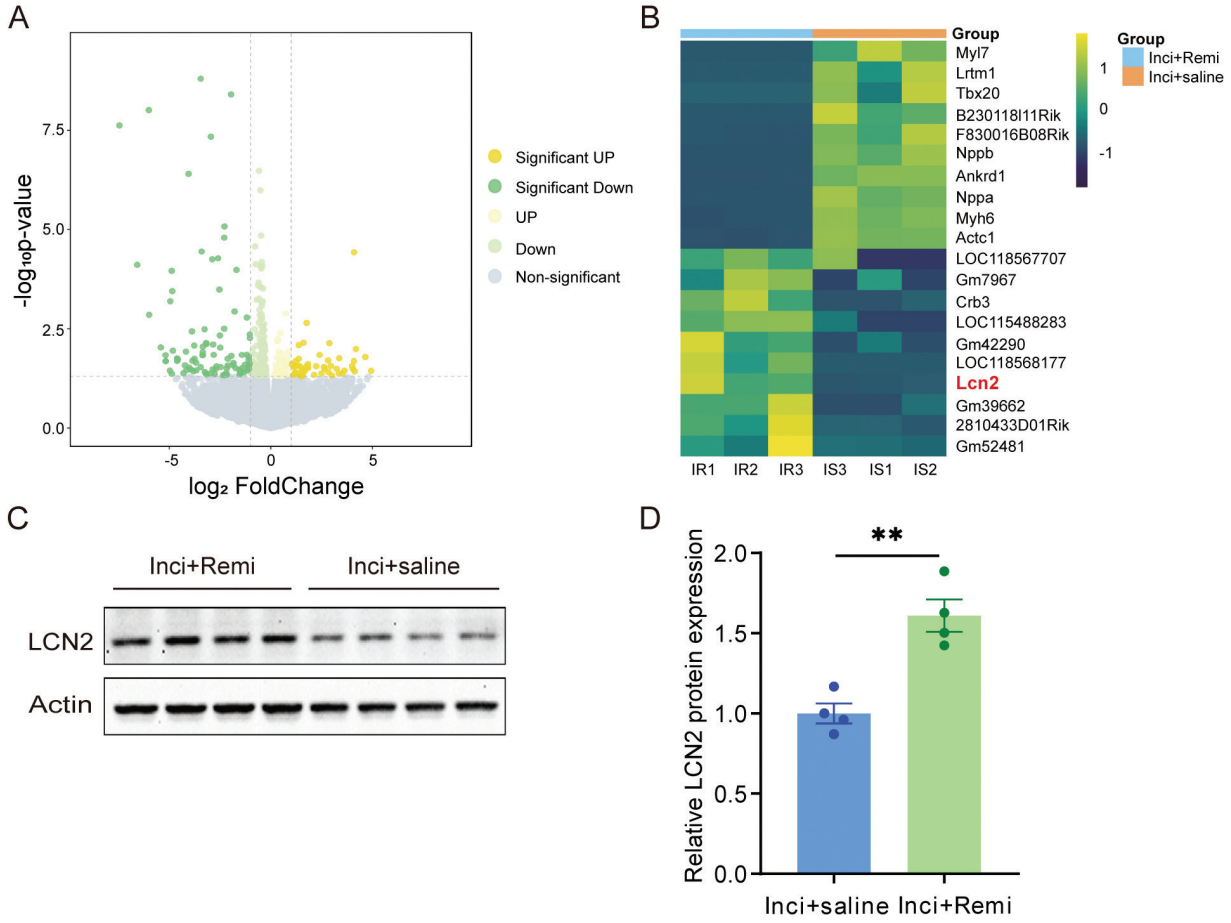

Fig. 2. *LCN2 expression was significantly increased in the ipsilateral ACC of RIPH mice. (A) Volcano plot of differentially expressed genes between inci + saline mice and inci + remi mice at day one. Downregulation and upregulation are shown as green and yellow dots, respectively. (B) Analysis of high expression levels of differentially expressed genes. (C and D) LCN2 protein level in the ipsilateral ACC tissue. (D, n = 4 mice per group, t (6) = 5.152, P = 0.0021, 2-tailed unpaired t-test). Data are presented as mean ± SEM. **P < 0.01.*

the ACC could effectively alleviate pain perception in RIPH mice (Fig. 4E).

LCN2 Overexpression Induced Mechanical Hyperalgesia in Mice

To investigate whether changes in the LCN2 levels within the ACC could directly affect the pain sensitivity of naïve mice, we constructed a broad-spectrum virus that overexpressed LCN2. We injected it into the left ACC of naïve mice (Figs. 5A,5B). For 3 weeks after we injected AAV-CMV-LCN2-EGFP (AAV-LCN2) or AAV-CMV-EGFP (AAV-Ctrl) into the ACC, the WB analysis confirmed that LCN2 protein levels were significantly higher in LCN2-overexpressing mice than in mice infected with the AAV-Ctrl (Figs. 5C,5D). We found that overexpression of LCN2 in the left ACC by AAV-LCN2 could lead to mechanical hyperalgesia in the mice's right hind paws (Fig. 5E). However, we observed no difference in the thermal pain thresholds in the right hind paws between the 2 groups of mice (Fig. 5F). Therefore, we concluded that elevated LCN2 levels in the ACC could directly induce mechanical hyperalgesia in mice.

Discussion

Studies have shown that some opioids can induce postoperative hyperalgesia, which is often caused by the ultra-short-acting μ-opioid receptor agonist remifentanil. RIPH is the most significant and most intense form of hyperalgesia (2). Although current pharmacological interventions can alleviate remifentanil-induced

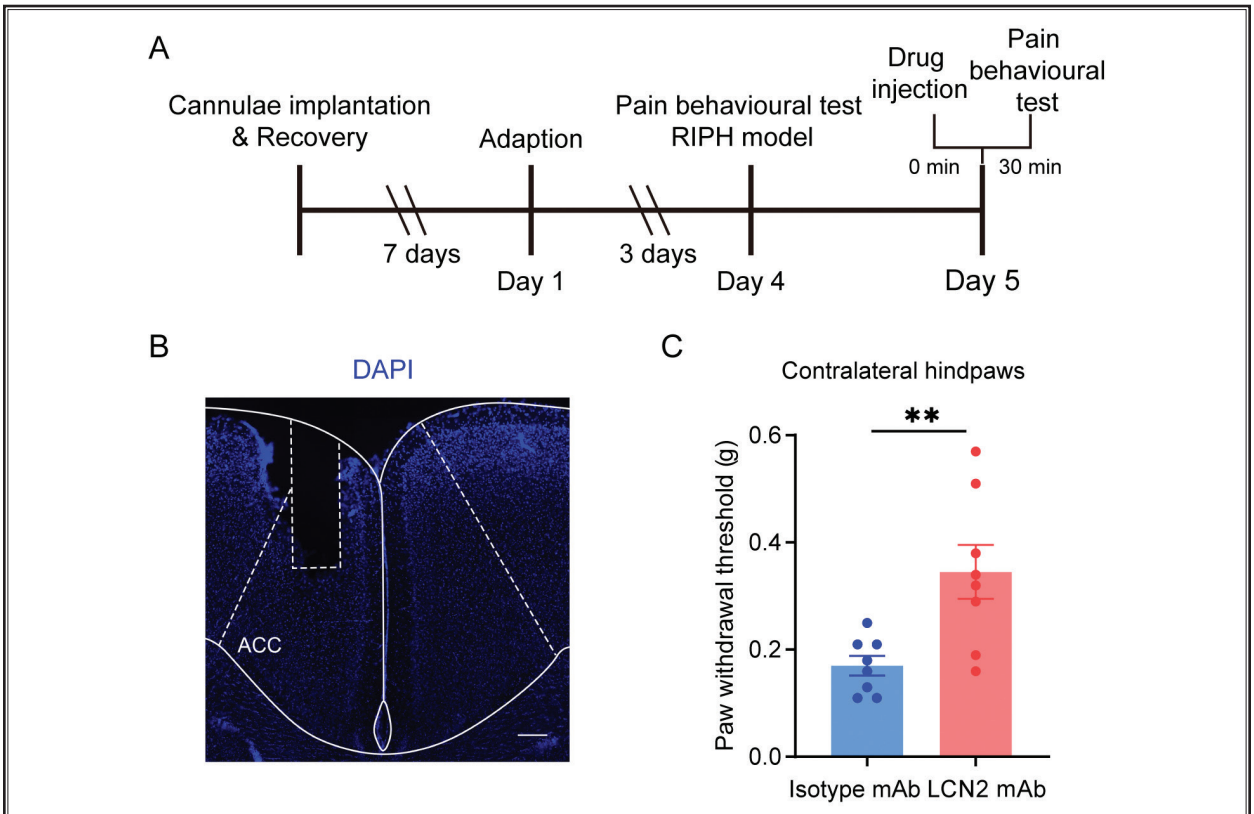

Fig. 3. *Intra-anterior cingulate cortex (ACC) injection of LCN2 mAb alleviated remifentanil-induced postoperative hyperalgesia in mice.*
*(A) Timeline of cannula implantation, LCN2 mAb administration, and pain behavioral test. (B) Representative brain slice of cannula implantation in ipsilateral ACC, scale bar = 500 μm. (C) Withdrawal threshold of mice in RIPH group after LCN2 mAb or isotype mAb injection (n = 8 mice per group, t(13.99) = 3.752, P = 0.0021, 2-tailed unpaired t-test with Welch's correction). Data are presented as mean ± SEM. **P < 0.01.*

OIH to some degree, they result in inevitable adverse effects (2). To improve the satisfaction and sense of gain of future surgical patients, we used a RIPH-mouse model to investigate the underlying molecular mechanisms of OIH. Our research found that the expression of LCN2 levels was significantly increased in the ipsilateral ACC of mice with mechanical hyperalgesia in the contralateral hind paws of the incision. Knockdown of LCN2 in ACC effectively alleviated mechanical hyperalgesia in RIPH mice.

More direct evidence is still needed from animal and clinical studies to minimize the impact of RIPH and decrease the use of other postoperative analgesic drugs. To explore the underlying molecular mechanisms of RIPH, we performed transcriptome sequencing on the ipsilateral ACC of RIPH mice and analyzed the results. We found that the expression level of LCN2 was significantly more upregulated in the RIPH mice than in the control group. Lipocalin-2 is a 24kDa secreted glycoprotein. In the central nervous system, LCN2 can participate in neuroimmune responses, regulate various cell phenotypes, and play a crucial role in developing biobehaviors such as anxiety, cognition, and pain sensitivity (30). Previous studies have found that the expression of LCN2 in the prefrontal cortex is significantly upregulated during inflammatory pain (31). Similarly, a recent study found that the level of LCN2 was significantly upregulated in a mouse model of chronic neuropathic pain induced by spinal nerve ligation (SNI) (22). These results are consistent with our findings that LCN2 increases significantly in RIPH mice.

We found that functionally manipulating LCN2 by injecting LCN2-neutralizing antibodies into the ACC or knocking down the ACC's LCN2 significantly attenuated mechanical RIPH, indicating that the upregulation of LCN2 plays a crucial role in the development of the con-

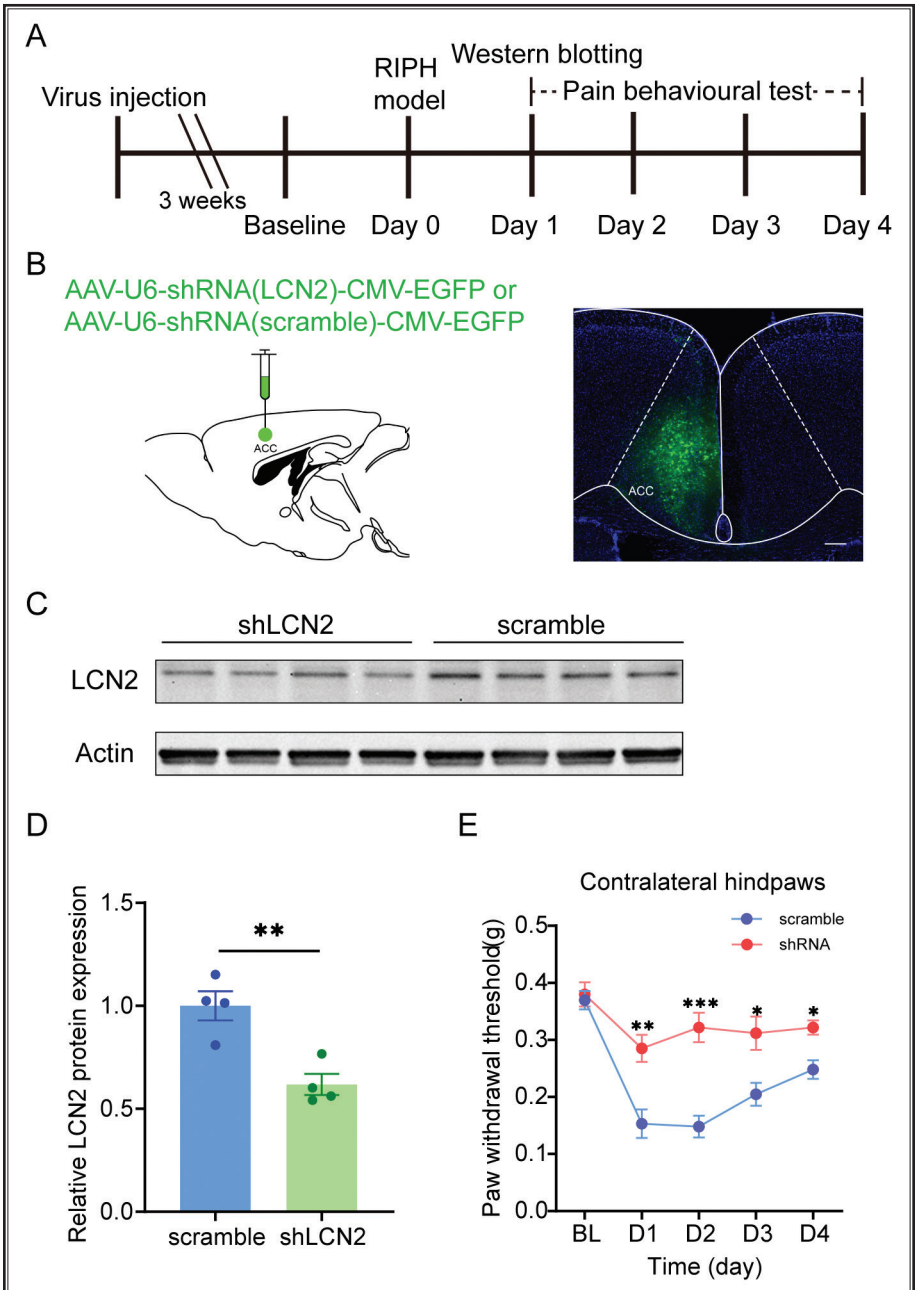

Fig. 4. *LCN2-knockdown-attenuated remifentanil-induced postoperative hyperalgesia. (A) Timeline of virus injection and pain behavioral test. (B) Left: schematic for a scramble or shLCN2 virus injection. Right: representative brain slice of AAV expression in the ipsilateral ACC, scale bar = 200 μm. (C and D) Western blotting of LCN2 expression in the left ACC of RIPH mice lysates after scramble or shLCN2 infusion (D, n = 4 mice per group, t (6) = 4.363, P = 0.0048, 2-tailed unpaired t-test). (E) Quantitative data for mechanical nociceptive thresholds (n = 10 mice per group, F(1,18) = 49.28, P < 0.0001). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.*

dition. Previous studies have reported that LCN2 protein is secreted by neurons (32) and glial cells (22). Earlier studies have also found that in the SNI mouse model, TRPV4 in the spinal cord mediates the activation and proliferation of microglia through the release of LCN2, which promotes synaptic transmission and plasticity of excitatory neurons, thereby affecting pain sensitivity (22). However, in the ACC of the RIPH-mouse model, it is unclear which cells secrete LCN2 and whether or how it affects neural plasticity. Therefore, the next step, is to continue exploring the cellular distribution of LCN2 and its mechanism of action.

In this study, we found that overexpressing LCN2 in the left ACC of naïve mice could directly induce mechanical hyperalgesia in the right hind paw without affecting thermal nociceptive thresholds. Previous studies have demonstrated that nociceptors and primary afferent fibers respond differently to thermal and mechanical stimuli. Eliminating specific subpopulations of nociceptors can lead to selective deficits in behavioral responses to specific noxious stimuli, demonstrating functional segregation of different nociceptor populations at the molecular level (33). Thus, in some cases, mechanical pain and thermal pain exhibit different response patterns, suggesting that these 2 types of pain may have distinct neural mechanisms.

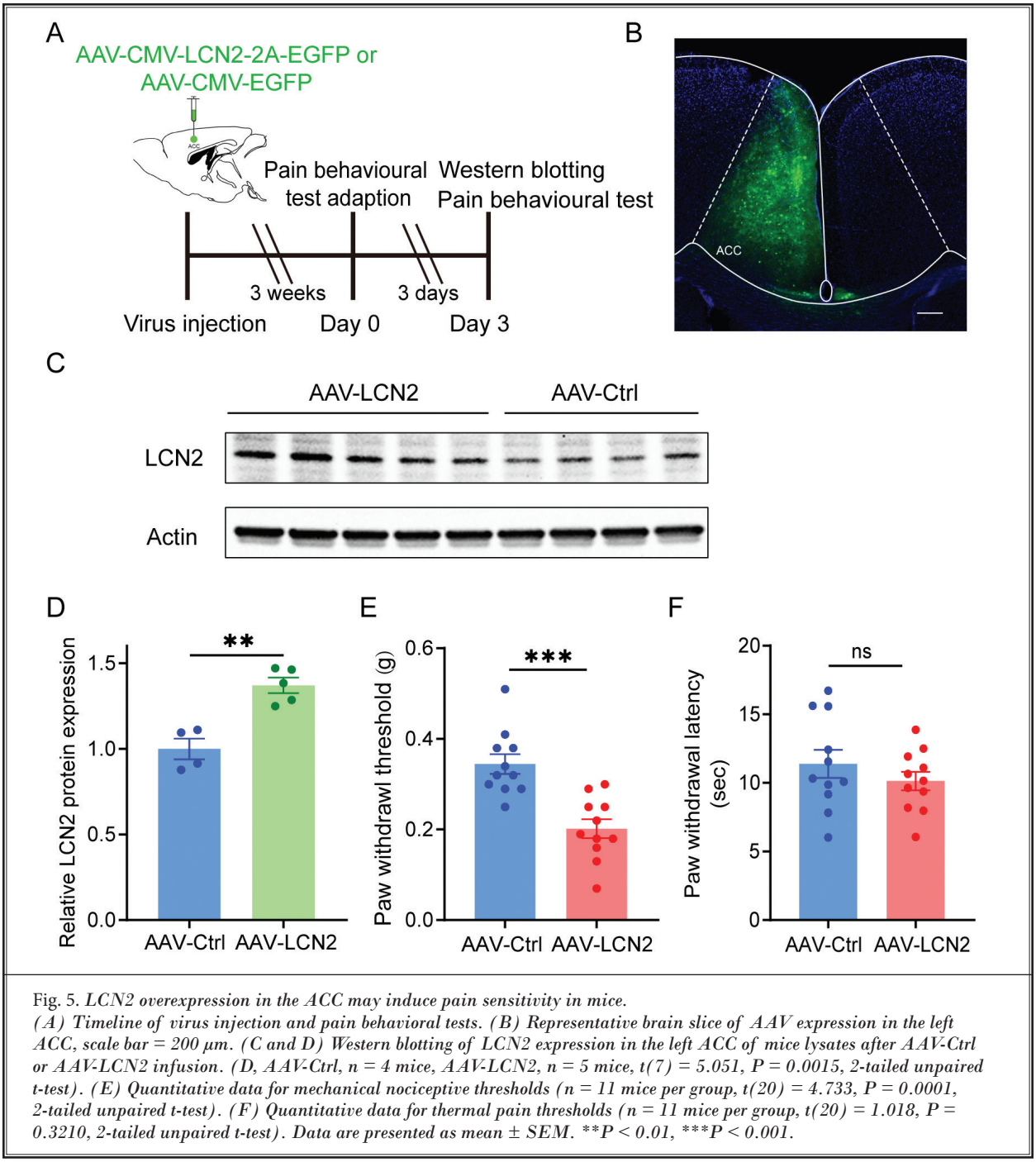

Fig. 5. *LCN2 overexpression in the ACC may induce pain sensitivity in mice.*
*(A) Timeline of virus injection and pain behavioral tests. (B) Representative brain slice of AAV expression in the left ACC, scale bar = 200 μm. (C and D) Western blotting of LCN2 expression in the left ACC of mice lysates after AAV-Ctrl or AAV-LCN2 infusion. (D, AAV-Ctrl, n = 4 mice, AAV-LCN2, n = 5 mice, t(7) = 5.051, P = 0.0015, 2-tailed unpaired t-test). (E) Quantitative data for mechanical nociceptive thresholds (n = 11 mice per group, t(20) = 4.733, P = 0.0001, 2-tailed unpaired t-test). (F) Quantitative data for thermal pain thresholds (n = 11 mice per group, t(20) = 1.018, P = 0.3210, 2-tailed unpaired t-test). Data are presented as mean ± SEM. **P < 0.01, ***P < 0.001.*

In summary, the upregulation of LCN2 in the ACC plays a crucial role in the development of RIPH. To some extent, RIPH may elicit concerns before or during the administration of perioperative analgesia. Given the critical role played by LCN2, targeting this protein for treatment has potential clinical value. Future studies are therefore needed to further reveal the specific mechanisms underlying the upregulation of LCN2 in RIPH and to investigate the regulatory mechanisms of LCN2. Also required are studies on new drug research and development and their safety and efficacy in combating postoperative hyperalgesia, thus contributing

to enhanced recovery after surgery and improving future surgical patients' satisfaction and sense of gain.

Limitations

This study focused only on the relationship between the upregulation of LCN2 expression and the occurrence of RIPH. We need to conduct further investigations into the effects of LCN2 upregulation on the activity of neurons, microglia, astrocytes, and subsequent impact on pain sensitivity to deepen our understanding of the underlying mechanisms involved. In addition, we also need many preclinical experiments to prove whether LCN2 can be an effective and safe therapeutic target.

Conclusions

This study indicated that the upregulation of LCN2 expression in the ACC could induce mechanical hyperalgesia in mice. LCN2 up-expression plays a crucial role in the occurrence of RIPH. This study has suggested that LCN2 may be a target for the treatment of the condition.

References

1. Weinbroum AA. Postoperative hyperalgesia-A clinically applicable narrative review. *Pharmacol Res* 2017; 120:188-205.
2. Wilder-Smith OH, Arendt-Nielsen L. Postoperative hyperalgesia: Its clinical importance and relevance. *Anesthesiology* 2006; 104:601-607.
3. Fletcher D, Martinez V. Opioid-induced hyperalgesia in patients after surgery: A systematic review and a meta-analysis. *Br J Anaesth* 2014; 112:991-1004.
4. Leal PC, Salomão R, Brunialti MKC, Sakata RK. Evaluation of the effect of ketamine on remifentanil-induced hyperalgesia: A double-blind, randomized study. *J Clin Anesth* 2015; 27:331-337.
5. Yu EH, Tran DH, Lam SW, Irwin MG. Remifentanil tolerance and hyperalgesia: Short-term gain, long-term pain? *Anaesthesia* 2016; 71:1347-1362.
6. Aguado D, Abreu M, Benito J, Garcia-Fernandez J, Gómez de Segura IA. Effects of naloxone on opioid-induced hyperalgesia and tolerance to remifentanil under sevoflurane anesthesia in rats. *Anesthesiology* 2013; 118:1160-1169.
7. Klimscha W, Ullrich R, Nasel C, et al. High-dose remifentanil does not impair cerebrovascular carbon dioxide reactivity in healthy male volunteers. *Anesthesiology* 2003; 99:834-840.
8. Song JW, Lee YW, Yoon KB, Park SJ, Shim YH. Magnesium sulfate prevents remifentanil-induced postoperative hyperalgesia in patients undergoing thyroidectomy. *Anesth Analg* 2011; 113:390-397.
9. Jin Y, Mao Y, Chen D, et al. Thalamocortical circuits drive remifentanil-induced postoperative hyperalgesia. *J Clin Invest* 2022; 132:e158742.
10. Kuner R, Kuner T. Cellular circuits in the brain and their modulation in acute and chronic pain. *Physiol Rev* 2021; 101:213-258.
11. Sun Y, Zhang W, Liu Y, Liu X, Ma Z, Gu X. Intrathecal injection of JWH015 attenuates remifentanil-induced postoperative hyperalgesia by inhibiting activation of spinal glia in a rat model. *Anesth Analg* 2014; 118:841-853.
12. Zeng J, Li S, Zhang C, Huang G, Yu C. The mechanism of hyperalgesia and anxiety induced by remifentanil: Phosphorylation of GluR1 receptors in the anterior cingulate cortex. *J Mol Neurosci* 2018; 65:93-101.
13. Zhou J, Qi F, Hu Z, et al. Dezocine attenuates the remifentanil-induced postoperative hyperalgesia by inhibition of phosphorylation of CaMKIIα. *Eur J Pharmacol* 2020; 869:172882.
14. Apkarian AV, Bushnell MC, Treede RD, Zubieta JK. Human brain mechanisms of pain perception and regulation in health and disease. *Eur J Pain* 2005; 9:463-484.
15. Bushnell MC, Ceko M, Low LA. Cognitive and emotional control of pain and its disruption in chronic pain. *Nat Rev Neurosci* 2013; 14:502-511.
16. Sprenger C, Eichler I-C, Eichler L, Zöllner C, Büchel C. Altered signaling in the descending pain-modulatory system after short-term infusion of the μ-opioid agonist remifentanil. *J Neurosci* 2018; 38:2454-2470.
17. Qi M, Li C, Li J, et al. Fluoxetine reverses hyperactivity of anterior cingulate cortex and attenuates chronic stress-induced hyperalgesia. *Neuropharmacology* 2022; 220:109259.
18. Yao PW, Wang SK, Chen SX, Xin WJ, Liu XG, Zang Y. Upregulation of tumor necrosis factor-alpha in the anterior cingulate cortex contributes to neuropathic pain and pain-associated aversion. *Neurobiol Dis* 2019; 130:104456.
19. Jung BK, Park Y, Yoon B, et al. Reduced secretion of LCN2 (lipocalin 2) from reactive astrocytes through autophagic and proteasomal regulation alleviates inflammatory stress and neuronal damage. *Autophagy* 2023; 19:2296-2317.
20. Jha MK, Jeon S, Jin M, et al. The pivotal role played by lipocalin-2 in chronic inflammatory pain. *Exp Neurol* 2014; 254:41-53.
21. Lee S, Park JY, Lee WH, et al. Lipocalin-2 is an autocrine mediator of reactive astrocytosis. *J Neurosci* 2009; 29:234-249.
22. Hu X, Du L, Liu S, et al. A TRPV4-dependent neuroimmune axis in the spinal cord promotes neuropathic pain. *J Clin Invest* 2023; 133:e161507.
23. Mucha M, Skrzypiec AE, Schiavon E, Attwood BK, Kucerova E, Pawlak R. Lipocalin-2 controls neuronal excitability and anxiety by regulating dendritic spine formation and maturation. *Proc Natl Acad Sci USA* 2011; 108:18436-18441.
24. Jeon S, Jha MK, Ock J, et al. Role of lipocalin-2-chemokine axis in the development of neuropathic pain following peripheral nerve injury. *J Biol Chem* 2013; 288:24116-24127.
25. Lee L, McLemore E, Rashidi L. Same-day discharge after minimally invasive colectomy. *JAMA Surg* 2022; 157:1059-1060.
26. Majholm B, Bartholdy J, Clausen HV, Virkus RA, Engbæk J, Møller AM. Comparison between local anaesthesia with remifentanil and total intravenous

anaesthesia for operative hysteroscopic procedures in day surgery. *Br J Anaesth* 2012; 108:245-253.

27. Pogatzki EM, Raja SN. A mouse model of incisional pain. *Anesthesiology* 2003; 99:1023-1027.
28. Huang C, Li H, Xu Y, et al. BICC1 drives pancreatic cancer progression by inducing VEGF-independent angiogenesis. *Signal Transduct Target Ther* 2023; 8:271.
29. Chen Q, Heinricher MM. Plasticity in the link between pain-transmitting and pain-modulating systems in acute and persistent inflammation. *J Neurosci* 2019; 39:2065-2079.
30. Jha MK, Lee S, Park DH, et al. Diverse functional roles of lipocalin-2 in the central nervous system. *Neurosci Biobehav Rev* 2015; 49:135-156.
31. Poh KW, Yeo JF, Stohler CS, Ong WY. Comprehensive gene expression profiling in the prefrontal cortex links immune activation and neutrophil infiltration to antinociception. *J Neurosci* 2012; 32:35-45.
32. Nagase T, Tohda C. Skeletal muscle atrophy-induced hemopexin accelerates onset of cognitive impairment in Alzheimer's disease. *J Cachexia Sarcopenia Muscle* 2021; 12:2199-2210.
33. Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and molecular mechanisms of pain. *Cell* 2009; 139:267-284.