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Peripheral Administration of an Opioid Peptide Analog Ameliorates Morphine-Produced Hyperalgesia in a Spared Nerve Injury Model

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Abstract

Traditional opioids have proven analgesic effects in clinical applications but are also associated with side effects, especially hyperalgesia. Reducing the occurrence of hyperalgesia is part of effective clinical pain management. In this study, we investigated the difference between MEL-0614, a novel endomorphin analog that can effectively penetrate the blood–brain barrier, and morphine in inducing hyperalgesia. Under mechanical and thermal stimulation conditions, intravenous administration of morphine led to hyperalgesia even at low concentrations, which was not observed with MEL-0614 even at high concentrations. In a spared nerve injury model, significantly less aggravation of allodynia was caused by an intravenous injection of MEL-0614 compared with that caused by morphine, and the allodynia symptoms occurred later. Notably, MEL-0614 significantly relieved the symptoms of morphine-induced allodynia. The activation of *N*-methyl-D-aspartic acid receptor and expression of inflammatory mediators differed in spinal microglia after intravenous injections of MEL-0614 and morphine. Intravenous injections of morphine induced increases in the number of microglia and overexpression of inflammatory factors, including tumor necrotic factor and interleukin-1β. Conversely, the effects of MEL-0614 administration did not differ from those of saline, and there was no inflammatory mediator overexpression. Especially in the spared nerve injury model, the cross-administration of morphine and MEL-0614 could reduce the expression of Toll-like receptor 4 and other related genes to different degrees compared with the use of morphine alone. Concurrently, the results could also explain the alleviating effect of MEL-0614 on morphine-induced pain sensitivity in behavioral experiments. Our findings may provide important information regarding the clinical treatment of neuropathic pain in the future.

Keywords Hyperalgesia · Neuropathic allodynia · Peripheral administration · Spared nerve injury · Opioid peptide

Abbreviations

ANOVA	Analysis of variance
AUC	Area under the curve
BBB	Blood–brain barrier
BDNF	Brain-derived growth factor
EMs	Endomorphins
IV	Intravenous
IL-1β	Interleukin-1β
NMDA	*N*-methyl-D-aspartic acid
P2X7R	P2X receptor 7
qPCR	Quantitative PCR
SNI	Spared nerve injury
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
WT	Withdrawal threshold

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Introduction

Pain is considered a universal social problem. Severe pain affects the life and work of patients, leading to marked production losses and high medical expenses. Among the various types of drugs used for pain treatment, opioids are considered the cornerstone for the treatment of moderate to severe pain, including postoperative, inflammatory, burn, and neuropathic pain. In clinical applications, traditional opioids such as morphine and fentanyl exert effective analgesic effects, but they also cause side effects such as dependence, addiction, respiratory depression, and constipation, resulting in considerable patient distress, which makes the clinical use of opioids a double-edged sword (Kim et al. 2015; Koller et al. 2019). Among the side effects, the phenomenon of opioid-induced hyperalgesia has received increasing attention from clinicians and pharmacologists (Khan and Mehan 2021; Yi and Pryzbylkowski 2015).

Neuropathic pain is caused by pathology or disease, affects the somatosensory system, and often occurs after surgery. Approximately 20% of patients worldwide experience nerve pain, which markedly affects their life (van Hecke et al. 2014). In recent years, an increasing number of studies have shown that regardless of whether the body is sick, opioid-induced allodynia will ensue. The use of opioids can aggravate the symptoms of neuropathic pain before taking medication and greatly extend the duration of pain (Grace et al. 2016; Starnowska-Sokol and Przewlocka 2020). Allodynia caused by opioids poses an enormous safety hazard, and the abuse of opioids by patients with hyperalgesia threatens their life and safety (Silverman 2009). Simultaneously, the abnormal pain caused by opioids greatly reduces patient medication compliance; therefore, research on hyperalgesia is particularly important.

Clinical and experimental studies have confirmed that after the use of opioids, patients may experience abnormal pain or hyperalgesia with an increased pain response (Mercadante et al. 2019). This manifests as a lowered pain threshold or a stronger pain response to weaker pain stimuli, and the occurrence of this symptom is not limited by the method of administration and the number of administrations. Numerous possible mechanisms of hyperalgesia have been proposed, including functional changes in opioid receptors, the role of endogenous neuropeptides, and enhancement of central glutamatergic system activity (da Cunha Leal et al. 2010; Roeckel et al. 2016). Moreover, it could be related to the activation of spinal microglia to release active substances related to pain conduction and pain modulation (Wen et al. 2011). Therefore, effectively reducing the occurrence of opioid-associated hyperalgesia is an integral part of clinically effective pain management.

Opioids are usually administered peripherally. Opioids can pass through the blood–brain barrier (BBB) and bind to central or peripheral opioid receptors (Chaves et al. 2017). In recent years, preclinical and clinical studies have shown that peripheral use of opioids for analgesia can lead to hyperalgesia (Compton et al. 2020; Zhang et al. 2015), and the manifestations of hyperalgesia are diverse, with slight temperature changes (heat or cold stimulation) and mechanical stimulation both reducing the body's ability to tolerate pain but to varying degrees (Abrahamsen et al. 2008; MacDonald et al. 2020). Some reports have shown that even ultra-low doses of opioids can cause hyperalgesia. These phenomena have led to difficulties in the clinical application of such drugs (Jensen and Finnerup 2014).

Two endogenous opioid peptides, endomorphin-1 (EM-1) and endomorphin-2 (EM-2), were isolated from bovine brains and human cerebral cortices in 1997, and showed the advantages of opioid receptor affinity, low toxicity, and minimal side effects; therefore, they held promise as potential analgesic drugs that could replace morphine (Przewlocki et al. 1999; Zadina et al. 1997). However, endomorphins are susceptible to enzymolysis, have difficulty penetrating the BBB, and have low bioavailability, which restricts their clinical application. Therefore, pharmacological efforts to modify them are ongoing (Bodnar 2021; De Marco and Janecka 2015; Gu et al. 2017; Varamini and Toth 2013). Our laboratory previously designed and synthesized a new type of EM-1 analog, MEL-0614, which exhibits the high analgesic ability of the parent peptide, while concurrently overcoming its defects and has good enzymatic stability, effectively penetrating the BBB to reach the central nervous system and exert analgesic effects (Liu et al. 2013; Wang et al. 2015). As hyperalgesia caused by opioids may occur both peripherally and centrally after administration, we previously reported research on hyperalgesia caused by its injection (Ma et al. 2020). This study further refines and studies whether peripheral administration of opioids can induce hyperalgesia. The main purpose of this study was to explore the difference in the induction of hyperalgesia between MEL-0614 and morphine in Kunming mice and spared nerve injury (SNI) model mice and assess the effect of the cross administration of morphine and MEL-0614 on hyperalgesia after intravenous (IV) administration.

Materials and Methods

Animals

The male Kunming and CX3CR1-GFP mice (18–22 g) used in the experiment were purchased from the Laboratory Animal Center of Lanzhou University. CX3CR1-GFP mice were obtained from the Jackson Laboratory in the United States.

The mice were housed in a designated standard animal room with an ambient temperature of 22 ± 1 °C and a 12-h light/dark cycle. Mice were allowed free access to water and food. The experiments conducted in this study met the ethical and moral requirements. To reduce the suffering of animals, each mouse was used only once. All experiments in this study were approved by the ethics committee of Lanzhou University (license number: SYXK Gan 2009-0005).

Drugs and Administration

The drugs and compounds used in the experiment were dissolved in 0.9% normal saline and stored at − 20 °C. Morphine hydrochloride was purchased from Shenyang No. 1 Pharmaceutical Factory (Shenyang, China). Sodium pentobarbital was purchased from Merck (Merck, Germany). Trizol, primers, reverse transcription kit, and SYBR fluorescent dye were purchased from TaKaRa Reagents (TaKaRa, Japan). MEL-0614 was synthesized using liquid-phase fragment condensation according to a previously reported method and purified by RP-HPLC (Liu et al. 2013). The purity of MEL-0614 was found to be > 98%.

IV injections were mainly used in the experiments. Suitable mice were selected and placed in a fixator. The tail was exposed, and unobstructed breathing was ensured. The tail of the mice was wiped with an alcohol cotton ball to help reveal the vein, and the drug was slowly injected into the vein with a 1 mL syringe. The injection volume for each mouse was 100 µL.

Modeling of the SNI Model

According to a previously reported experimental method (Decosterd and Woolf 2000; Richner et al. 2011), mice were anesthetized through intraperitoneal injection of sodium pentobarbital (60 mg/kg), the sciatic nerve of the left leg of the mouse was exposed, the tibial and common peroneal nerves were located, 10–0 absorbable surgical sutures were used for ligation and 2–4 mm of the distal nerve was removed, leaving the sural nerve. The wound was treated with 4–0 surgical sutures, and penicillin powder was sprinkled on the wound to prevent infection. The postoperative recovery period was 14 days, during which the mice were supplied with adequate food and water. The pain threshold of the mice was the lowest on the 14th day after the operation, therefore, this was the day selected to conduct the experiments.

von Frey Probe Mechanical Stimulation Experiment

According to a previously reported experimental method (Bonin et al. 2014; Ma et al. 2020), based on the results of the preliminary experiment, a 1.4-g von Frey probe was selected with the percentage of non-responsive stimuli produced being approximately 80%. Before starting the experiment, the mice were placed in a transparent box with a steel mesh at the bottom to adapt for 30 min. The plantar surface of the hind paw of the mouse was stimulated with a 1.4-g von Frey filament. Each mouse was tested ten times at an interval of 10 min each time, and the bending time of the filament was maintained for 2 s. A paw-raising or paw-flicking reaction was regarded as positive. The number of positive reactions in each mouse was recorded in ten tests. The experimental result was recorded as non-response to stimulation % = (number of non-responses/10 × 100).

Before the mechanical allodynia test in the SNI model, the mice were placed in a transparent plexiglass box to adapt for 30 min. von Frey probe (0.004–6.0 g) were used to stimulate the hind feet of the mice. When the mouse had a foot-withdrawal response, we recorded the number of g of the von Frey probe at that time. If the mouse did not have a foot withdrawal reaction, we used the next g of the von Frey probe to continue testing until the mouse had a positive reaction. To avoid tissue damage, the maximum test value allowed was 6.0 g. The same and contralateral sides of the operation were tested five times, and the average value was recorded. The experimental results were recorded as withdrawal threshold (WT) values.

Warm Bath Tail Flick Thermal Stimulation Experiment

According to a previously reported experimental method (Elhabazi et al. 2014), one-third of the tails of mice were placed in a constant temperature water bath at 48 ± 0.2 °C, and the time when the tails were out of the water was recorded as the control incubation period. Each mouse was tested three times at an interval of 10 min. The tail flapping time was recorded again after administration and was considered the test latency. To avoid tissue damage, we ensured that the test time did not exceed 25 s. The experimental results were recorded as the time of the tail flick.

RNA Isolation, Reverse Transcription, and Real-Time Quantitative PCR

The mouse spinal cord L4–L6 segment was extracted according to a previously reported experimental method (Ma et al. 2020), and the TRIzol reagent was used to extract total RNA. According to the instructions of the reverse transcription kit, we incubated at 37 °C for 15 min to reverse transcribe mRNA into cDNA. The SYBR kit and real-time fluorescence quantification system ABI QuantStudio 5 (ThermoFisher) were used for cDNA amplification. The PCR reaction conditions were 95 °C for 30 s, 95 °C for 5 s, 60 °C for 30 s, 40 cycles, and melting from 60 to 95 °C. The

Ct value of each gene was recorded, and we use the $2^{-\Delta\Delta ct}$ method for gene quantification. *GAPDH* was used as the reference gene. The primer sequences (Genbank, www.ncbi.nlm.nih.gov) are shown in Supplementary Table S1.

Immunofluorescence Detection

The microglia of the transgenic CX3CR1-GFP mouse exhibited autofluorescence. SNI-surgery CX3CR1-GFP mice and normal CX3CR1-GFP mice received continuous injection for 8 and 4 days, respectively. The L4–L6 spinal cord segments were extracted, fixed in 4% paraformaldehyde for 8 h, and placed in 20% sucrose. The solution was dehydrated overnight at 4 °C. The next day, the tissue was removed and embedded with an OTC embedding agent, and then the embedded tissue was placed in the freezer of a frozen slicer (Leica CM1950, Germany). After the tissue was frozen, the spinal cord section (thickness 10 μm) was placed on a slide. The slides were immersed in phosphate buffered saline buffer for 10 min to wash off the OCT embedding agent. The cells were then immediately observed under a fluorescence microscope (BX53; Olympus, Tokyo, Japan). After photographs were obtained, ImageJ was used for area normalization and quantification.

Statistical Analyses

SPSS 25.0 was used for statistical analysis. All values are reported as the mean ± standard error of the mean. There were no fewer than six mice in each behavioral experiment group. Two-way analysis of variance and Tukey's honest significant difference tests were used to detect statistically significant differences between experimental groups. One-way analysis of variance followed by Tukey's honest significant difference tests were used for the qPCR and immunofluorescence experiments. ImageJ was used for the quantitative analysis of fluorescent slices. Statistical significance was set at $P < 0.05$.

Results

Detection of Hyperalgesia After IV Injection

Kunming mice were treated with IV administered compounds for 4 consecutive days, followed by mechanical pain detection, and the probability of non-lifting was calculated by stimulating the hindfoot ten times with a 1.4-g von Frey probe. The experimental results are shown in Fig. 1a. Mice showed hyperalgesia on day 1 after a high concentration of 10 mg/kg morphine was administered ($P < 0.01$) and their symptoms increased over the following 3 days. The probability of non-lifting performance decreased to 45% ± 3.12 compared to 83.33% ± 1.92 in the saline group. In addition, there was a significant difference on the 4th day in the low (1 mg/kg) and medium (3 mg/kg) concentrations of morphine ($P < 0.001$). The mechanical hyperalgesia induced by morphine did not disappear at the end of administration. In contrast, at a high concentration of 10 mg/kg MEL-0614, mice developed mild pain sensitivity on the 4th day ($P < 0.05$), and the probability of non-lifting decreased to 71.67% ± 4.36. There were no significant differences for the MEL-0614 compounds at low (1 mg/kg) and medium (3 mg/kg) concentrations. A comprehensive comparison of area

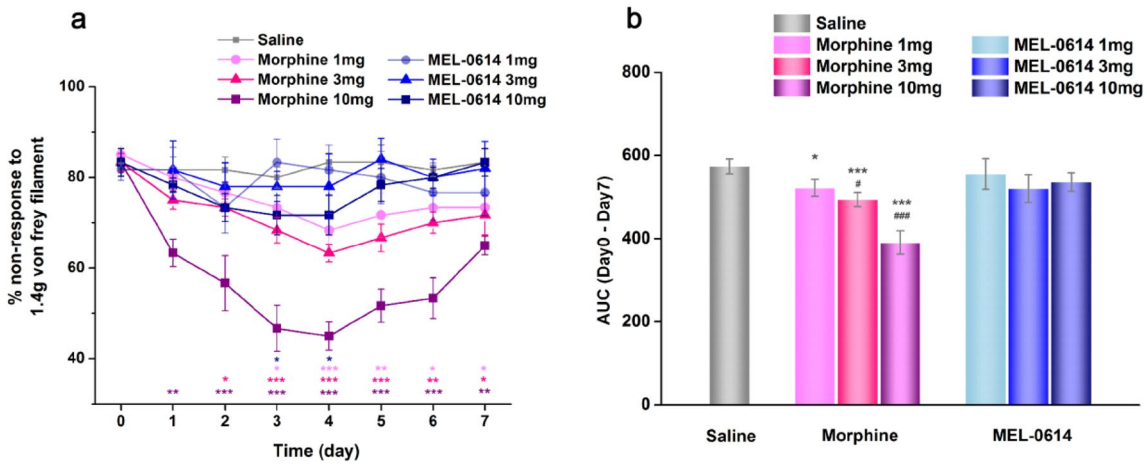

Fig. 1 Dose–response curve of mechanical stimulation hyperalgesia induced by IV administration of saline, morphine and MEL-0614 in normal mice (**a**). Area under the curve (AUC) data from day 0 to day 7 after IV administration (**b**). Doses used are shown in the figure and values are means ± standard error of the mean (SEM) of 6–10 mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicates that response was significantly different from saline, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ indicates that response was significantly different from MEL-0614 at the same dose according to two-way ANOVA followed by Tukey's HSD test

under the curve (AUC) data from day 0 to day 7 is shown in Fig. 1b, the AUC value of MEL-0614 at high concentration (10 mg/kg) was 536.67 ± 22.3, approximately 1.37 times higher than that in the morphine group (390.83 ± 28.1). Similarly, the mechanical hyperalgesia induced by morphine was significantly different from that induced by saline at low (1 mg/kg) and medium (3 mg/kg) concentrations, while no significant difference was observed for MEL-0614 at high, medium, and low concentrations.

The thermal stimulation model was tested using a warm bath tail flick experiment, and the experimental results are shown in Fig. 2a. After receiving a high concentration of 10 mg/kg MEL-0614 compound, the mice did not show mild hyperalgesia until the 4th day, and the duration of the base value decreased from 6.90 ± 0.48 s to 5.09 ± 0.21 s. However, hyperalgesia recurred quickly on the 5th day when the drug was stopped. There was no significant difference between the MEL-0614 compound and saline groups at low (1 mg/kg) and medium (3 mg/kg) concentrations. In the morphine group, the mice showed significant hyperalgesia from 6.83 ± 0.39 s to 5.98 ± 0.34 s ($P < 0.001$) on the 2nd day after receiving a high concentration of 10 mg/kg, and their symptoms increased over the following days. After 4 days of consecutive injections, the thermal stimulation hyperalgesia was most severe, and the base time of thermal stimulation decreased to 3.81 ± 0.20 s; this significant difference did not recover after the withdrawal of morphine. The same occurred at the low (1 mg/kg) and medium (3 mg/kg) concentrations. Comparing the AUC values of day 0 to day 7 (Fig. 2b), the AUC of MEL-0614 was 42.58 ± 1.78 at high concentration (10 mg/kg), approximately 1.20 times higher than that of morphine (35.54 ± 1.65). At the same low (1 mg/kg) and medium (3 mg/kg) concentrations, the thermal stimulation hyperalgesia induced by morphine was significantly different from that in the saline group, while that of MEL-0614 was not significantly different.

Detection of Neuropathic Allodynia Development in SNI Model Mice After IV Injection

The von Frey probe was used to test the abnormal sensation of mechanical stimulation in mice after administration. As shown in Fig. 3a, significant allodynia was observed 2 days after IV administration of 10 mg/kg morphine. After 8 days of continuous administration, the WT value on the operative side decreased from 0.46 ± 0.02 g to 0.10 ± 0.01 g. Moreover, this symptom was not limited to the surgical side. The WT value of the SNI model mice on the contralateral side also showed a significant decrease, from 2.58 ± 0.17 g to 0.42 ± 0.05 g (Fig. 3c). Regardless of whether morphine was administered for 4 or 8 days, the resulting dysphoria was still present at 14 days. In contrast, after IV administration of 10 mg/kg MEL-0614 in SNI model mice, the operative side group only presented mild allodynia symptoms (WT = 0.31 ± 0.03 g) on the 8th day of administration, and the symptoms disappeared with the discontinuation of the drug. No hyperalgesia occurred on the contralateral side. The effect of MEL-0614 on morphine-induced allodynia was investigated using cross-administration. In terms of cross-administration, the symptoms of abnormal pain sensation after 4 days of morphine (Treatment A) administration was somewhat recovered after using MEL-0614 (Treatment B), and the WT value of mice in the morphine/MEL-0614 group significantly increased after 4 days of MEL-0614

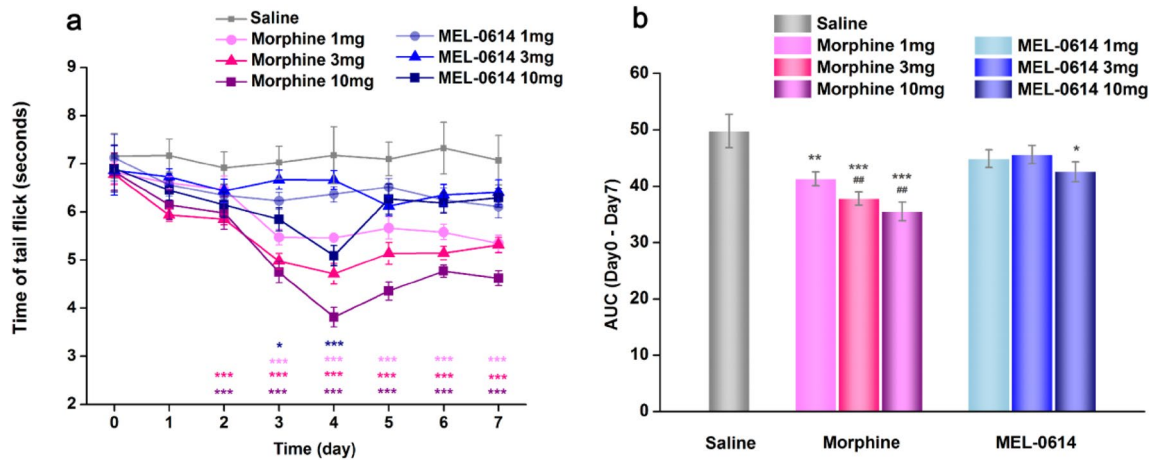

Fig. 2 Dose–response curve of thermal stimulation hyperalgesia induced by IV administration of saline, morphine and MEL-0614 in normal mice (**a**). AUC data from day 0 to day 7 after IV administration (**b**). Doses used are shown in the figure and values are means ± SEM of 6–10 mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicates that response was significantly different from saline, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ indicates that response was significantly different from MEL-0614 at the same dose according to two-way ANOVA followed by Tukey's HSD test

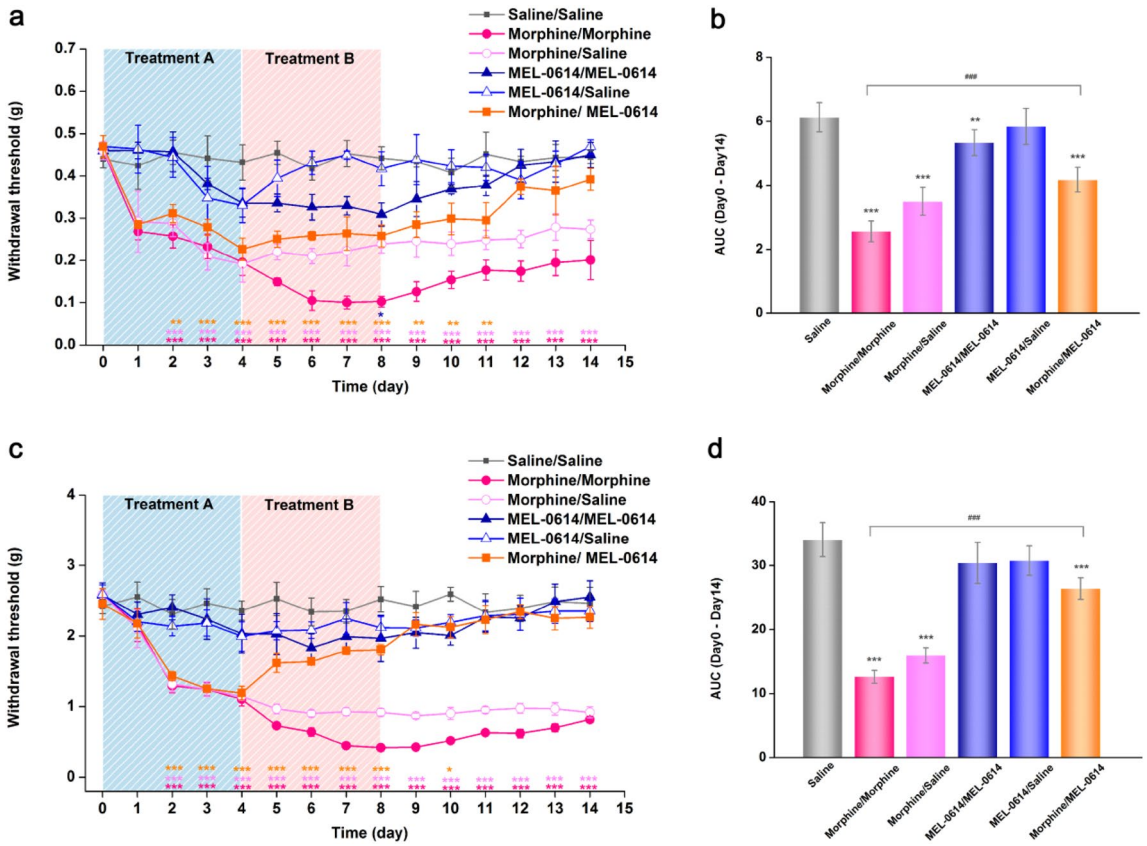

Fig. 3 The curve of allodynia in the ipsilateral side (**a**) and contralateral side (**c**) of SNI modle mice after IV injection. AUC data from day 0 to day 14 after IV administration (**b**, **d**). Dosage regimens used are shown in the figure. Treatment A plan take IV administration of the first drug for 4 days, and treatment B plan take IV administration of the second drug for 4 days. Values are means ± SEM of 6–10 mice. $^{**}P < 0.01$, $^{***}P < 0.001$ indicates that response was significantly different from saline, $^{\#\#\#}P < 0.001$ indicates that response was significantly different from morphine/MEL-0614 group according to two-way ANOVA followed by Tukey's HSD test

administration. The contralateral performance was particularly obvious, as the value increased from 1.19 ± 0.10 g to 1.81 ± 0.08 g. This indicates that MEL-0614 has a certain relief effect on morphine-induced allodynia. The AUCs of 0–14 days were compared (Fig. 3b, d). After 8 days of morphine administration, the AUCs of the operative and contralateral groups were 2.57 ± 0.32 and 12.6 ± 1.03, respectively. After cross-administration of morphine/MEL-0614, the AUC increased to 4.18 ± 0.38 and 26.41 ± 1.69, respectively. There was a significant difference compared to the corresponding control group ($P < 0.001$).

Under the thermal stimulation of the warm bath tail flick model, the SNI mice also showed symptoms of increased allodynia after continuous injection of 10 mg/kg morphine for 2 days. As shown in Fig. 4a, mice that received continuous IV injection of this concentration of morphine for 8 days experienced the most severe allodynia, and their WT value for 48 °C heat stimulation decreased from 6.05 ± 0.24 s to 3.43 ± 0.23 s. The allodynia caused by this dosing regimen remained present after the drug was discontinued and was maintained until the 14th day. A certain degree of recovery was observed in the morphine/saline group mice, who received saline injection after 4 days of continuous morphine injection, but there was a significant difference 10 days after the drug was discontinued. After 8 days of continuous MEL-0614 use, the results showed that the thermal-stimulation allodynia of the model mice was as low as 4.68 ± 0.17 s. After 4 days of consecutive administration the drug was discontinued; MEL-0614/saline group mice only developed mild allodynia symptoms on the 4th day. The allodynia symptoms of SNI mice immediately disappeared once MEL-0614 administration was stopped. Notably, when 10 mg/kg morphine was continuously used for 4 days and then changed to the same dose of MEL-0614, there was significant relief of allodynia symptoms and they returned to the normal saline level 1 day after the medication was discontinued. This indicates that MEL-0614 has a certain alleviating effect on the aggravation of morphine-induced neuralgesia. The AUC values of 0–14 days were compared (Fig. 4b); the AUC value of continuous administration of morphine for 8 days was 60.45 ± 2.19. The AUC value of the same administration of morphine/MEL-0614

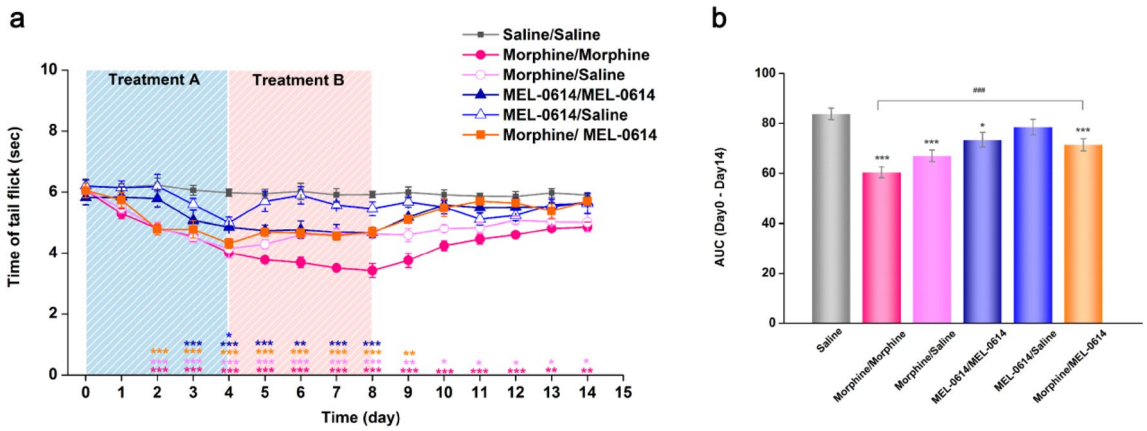

Fig. 4 The curve of thermal stimulation allodynia of SNI modle mice after IV injection (**a**). AUC data from day 0 to day 14 after IV administration (**b**). Dosage regimens used are shown in the figure. Treatment A plan take IV administration of the first drug for 4 days, and treatment B plan take IV administration of the second drug for 4 days. Values are means ± SEM of 6–10 mice. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ indicates that response was significantly different from saline, $^{\#\#\#}P<0.001$ indicates that response was significantly different from morphine/MEL-0614 group according to two-way ANOVA followed by Tukey's HSD test

was significantly different compared to the morphine/morphine group at 71.45 ± 2.46.

Expression of NMDA Receptors

The effect of NMDA receptor activation on hyperalgesia was investigated using qPCR. As shown in Fig. 5a, after IV administration for 4 days in normal mice, spinal RNA was extracted from the L4–L6 segments. It was found that *Grin2b*, an editing gene of NMDA receptor subtype NR2B, was significantly increased in the spinal cord of mice in the 10 mg/kg morphine group, and its expression level was 2.13 ± 0.13, 2.22 times that of the saline group. However, MEL-0614 at the same concentration did not significantly increase the expression level of *Grin2b* in the spinal cord of mice, and the ratio of the MEL-0614 group to saline group was 1.23. The amount of *Grin2b* in the spinal cord of SNI model mice was also measured after 8 days of continuous IV administration (Fig. 5b). The expression level of *Grin2b* in the 10 mg/kg morphine group was 3.10 times that of the saline group. The expression level of *Grin2b* in the spinal cord of the MEL-0614 group was 1.12 times that of the

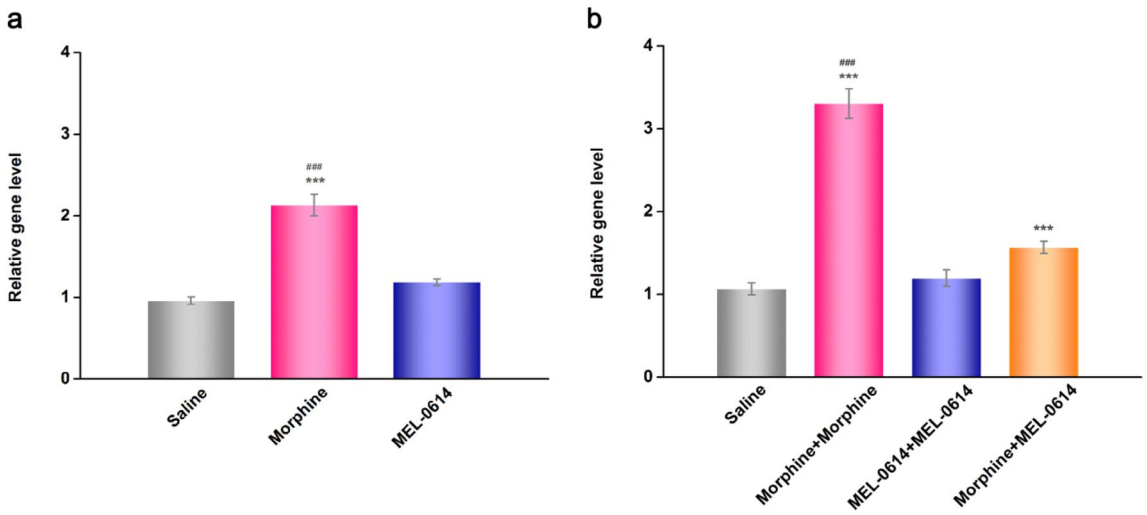

Fig. 5 The expression level of *Grin2b* gene of NMDA receptor in the spinal cord of normal mice (**a**) and SNI modle mice (**b**) after IV administration. Dosage regimens used are shown in the figure and values are means ± SEM of 6–12 mice. $^{***}P<0.001$ indicates that response was significantly different from saline, $^{\#\#\#}P<0.001$ indicates that response was significantly different from MEL-0614 according to one-way ANOVA followed by Tukey's HSD test

saline group, and there was no significant change compared with the value before administration. In the behavioral experiments, the crossover trial of morphine and MEL-0614 resulted in reduction in morphine-induced hyperalgesia, and the same result was found in the molecular experiments. Compared with morphine alone, cross-administration of morphine/MEL-0614 (morphine for 4 days and MEL-0614 for 4 days) reduced the expression level of *Grin2b* in the spinal cord of mice from 3.31 ± 0.18 to 1.57 ± 0.07.

Expression of Related Genes in Microglia

The effects of morphine and MEL-0614 on microglia were detected using qPCR and fluorescence section assays in CX3CR1-GFP mice. As shown in Fig. 6, the expression level of promoter TLR4 in spinal cord microglia cells was significantly increased in normal mice after IV administration of morphine for 4 days and was 1.73 times that of the saline group, showing a significant difference. The expression level of TLR4 in the IV administration MEL-0614 group was 1.14 times that in the saline group, and there was no significant difference. In addition, the expression levels of P2X4 receptors and BDNF in the spinal cord of normal mice nearly doubled after morphine injection, increasing by 1.90 and 1.96 times, respectively, compared with the saline group. The genes encoding the P2X4 receptor and BNDF in the MEL-0614 group were maintained at the same level as those in the saline group. The expression of TNF in the spinal cord of the morphine group was 2.12 times that of the saline group, and the content of IL-1β was 1.79 times that of the control group. After injection of MEL-0614, there was no significant difference in the inflammatory mediator content between the saline and control groups.

The fluorescence data more directly showed the number of microglia in the spinal cord of normal mice after drug injection. As shown in Fig. 7, the number of microglia in the L4–L6 segment of the spinal cord was the highest after continuous morphine injection, and that the quantitative data describing the microglia positive areas % in the spinal cord was $15.47\% \pm 1.52$. The value in the MEL-0614 group was not significantly different from that in the saline group, at $5.67\% \pm 0.43$ and $4.83\% \pm 0.54$, respectively.

The results of the qPCR and spinal cord fluorescence section experiments carried out on SNI mice are shown

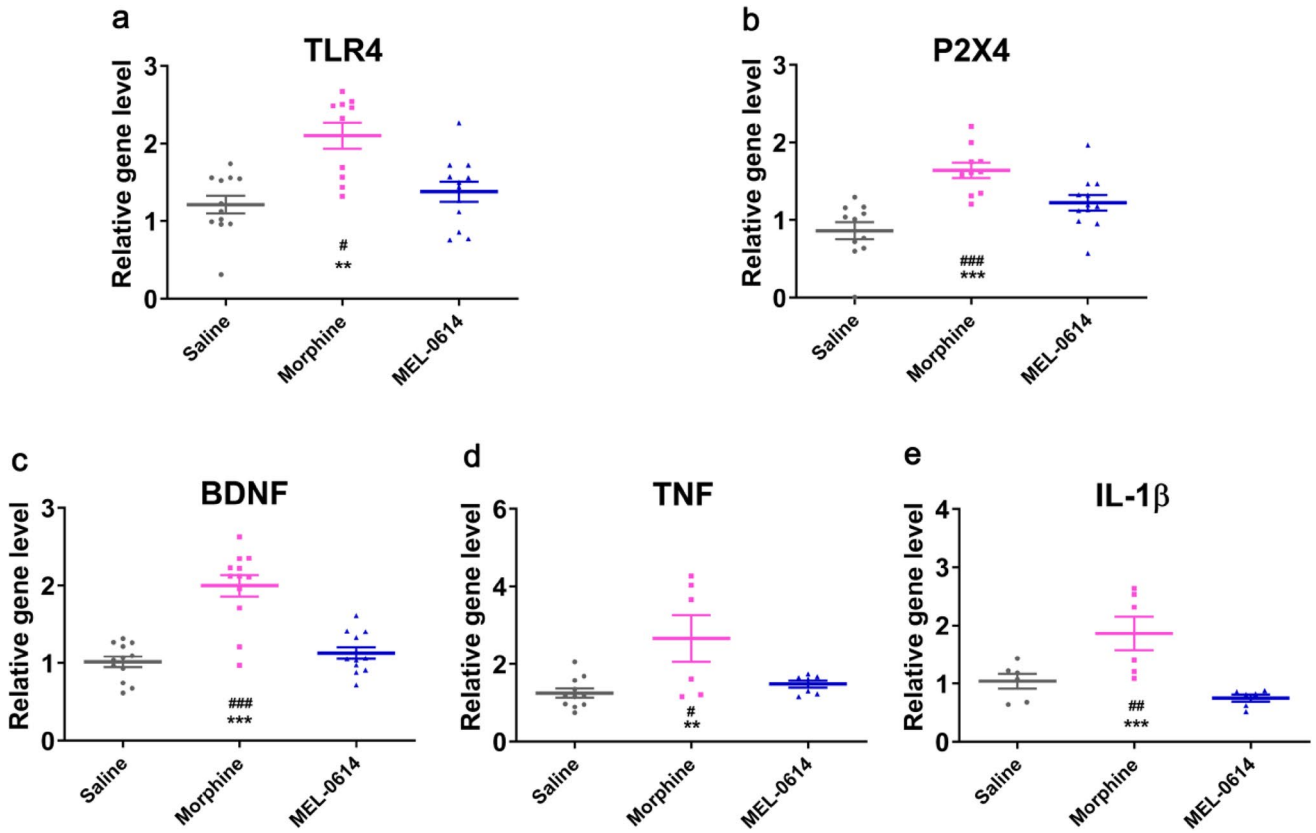

Fig. 6 The expression level of TLR4 (**a**), P2X4R (**b**), BDNF (**c**), TNF (**d**), and IL-1β (**e**) gene in the spinal cord of normal mice after IV administration. Dosage regimens used are shown in the figure and values are means ± SEM of 6–12 mice. $^{**}P < 0.01$, $^{***}P < 0.001$ indicates that response was significantly different from saline, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ indicates that response was significantly different from MEL-0614 according to one-way ANOVA followed by Tukey's HSD test

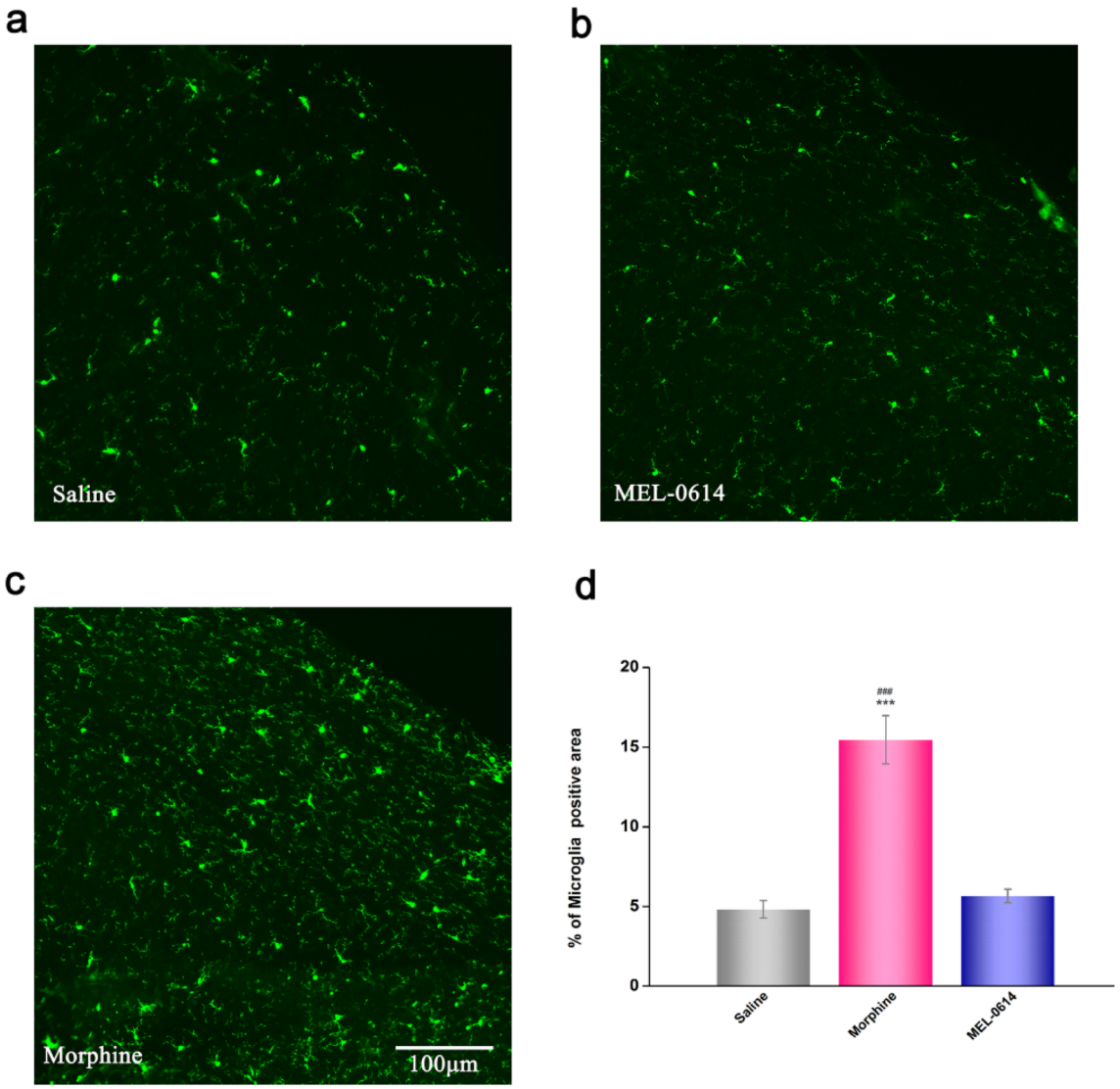

Fig. 7 The expression level of microglia in the spinal cord of normal mice after IV administration of saline (**a**), MEL-0614 (**b**) and morphine (**c**), bar = 100 μm, magnification: (×10). The quantitative data describing the microglia positive areas % in the spinal cord (**d**). Data are means ± SEM of 6–12 mice. $^{***}P < 0.001$ indicates that response was significantly different from saline, $^{###}P < 0.001$ indicates that response was significantly different from MEL-0614 according to one-way ANOVA followed by Tukey's HSD test

in Fig. 8. After 8 days of IV administration of morphine, the spinal cord L4–L6 was extracted, and the expression of various receptors and inflammatory mediators in the spinal cord increased to varying degrees. The expression of TLR4 was 3.01 times than that of the saline group, and the P2X4 receptor and BDNF were also overexpressed by 2.75 and 3.12 times, respectively. The inflammatory mediators TNF and IL-1β in the spinal cord were also over-released, and their expression levels were 4.33 and 4.08 times those of the saline group, respectively. In the IV administration MEL-0614 group, there was no significant change in the expression of the above receptors and factors. The expression level of TLR4 was 1.23 times that of the saline group, and the P2X4 receptor and BNDF remained at the same level as those in the saline group. Considering the performance of the cross-administration of morphine and MEL-0614 in the behavioral experiments, the expression of the morphine/MEL-0614 cross-administration group in the spinal cord of SNI mice was further tested. The results showed that TLR4 expression decreased to 2.45 times compare with morphine alone. The expression levels of other genes also decreased at different degrees.

The fluorescence results of SNI mice are shown in Fig. 9. The number of microglia in the spinal cord L4–L6 segment of SNI mice after continuous morphine injection showed the affected area was 19.45% ± 1.45, 2.62 times the affected area in the MEL-0614 group (7.41% ± 0.62). After the cross-administration of MEL-0614 and morphine, the affected area

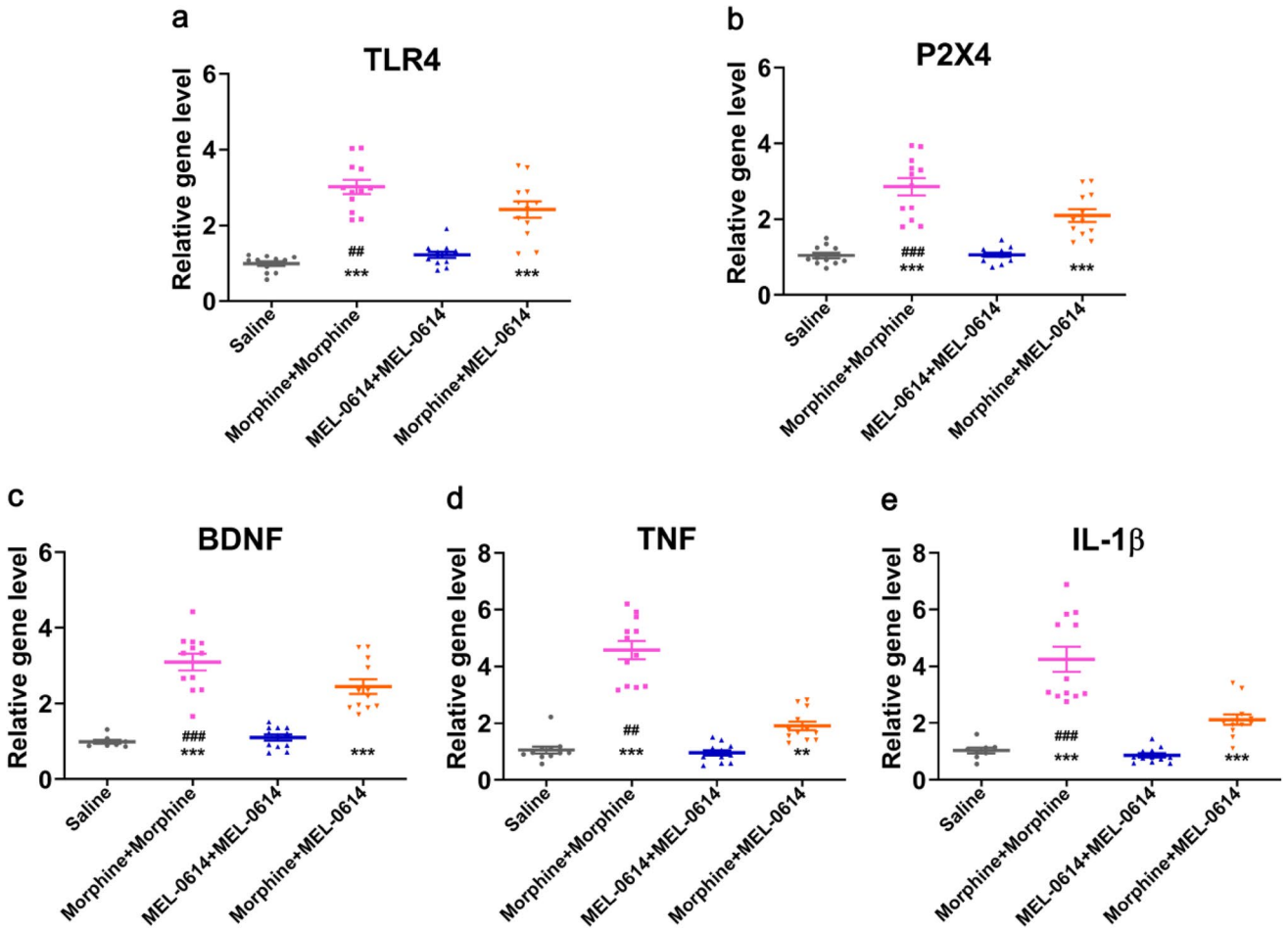

Fig. 8 The expression level of TLR4 (**a**), P2X4R (**b**), BDNF (**c**), TNF (**d**), and IL-1β (**e**) gene in the spinal cord of SNI modle mice after IV administration. Dosage regimens used are shown in the figure and values are means ± SEM of 6–12 mice. $^{**}P<0.01$, $^{***}P<0.001$ indicates that response was significantly different from saline, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ indicates that response was significantly different from MEL-0614 according to one-way ANOVA followed by Tukey's HSD test

of microglia in the spinal cord section was 7.03% ± 0.75, 0.36 times that of morphine alone (Fig. 9e).

Discussion and Conclusion

Morphine is the most widely used drug for the clinical treatment of pain but is also associated with certain side effects, one of which is hyperalgesia (Lee et al. 2011). MEL-0614 is an endomorphine analog that was independently designed and synthesized by our research team (Liu et al. 2013). Previous studies have shown that MEL-0614 has a highly effective analgesic effect and can penetrate the BBB (Cui et al. 2020; Wang et al. 2015; Zhou et al. 2021). Therefore, we investigated the differences between morphine and MEL-0614 in the production of pain sensitivity at the behavioral and molecular levels.

MEL-0614 shows a good effect during peripheral injection and can cross the BBB, indicating its potential clinical utility. It has been reported that opioids and morphine can relieve tolerance and opioid-induced hyperalgesia without affecting the analgesic effect in a neuropathic model (Corder et al. 2017). Therefore, we speculated that the BBB permeability of the compound would affect the analgesic effect, and the common adverse reactions of opioids. It is not easy to induce hyperalgesia if the main action site of the compound is central and the BBB permeability is sufficient.

In a study of hyperalgesia in normal mice, peripherally injected compounds could induce hyperalgesia in a dose-dependent manner under mechanical and thermal stimulation conditions. When assessing for hyperalgesia after IV injection, a 1.4-g von Frey probe was used to provide mechanical stimulation to the hindfoot of mice. Continuous administration of morphine induced hyperalgesia at high, medium, and low concentrations. The symptoms continued

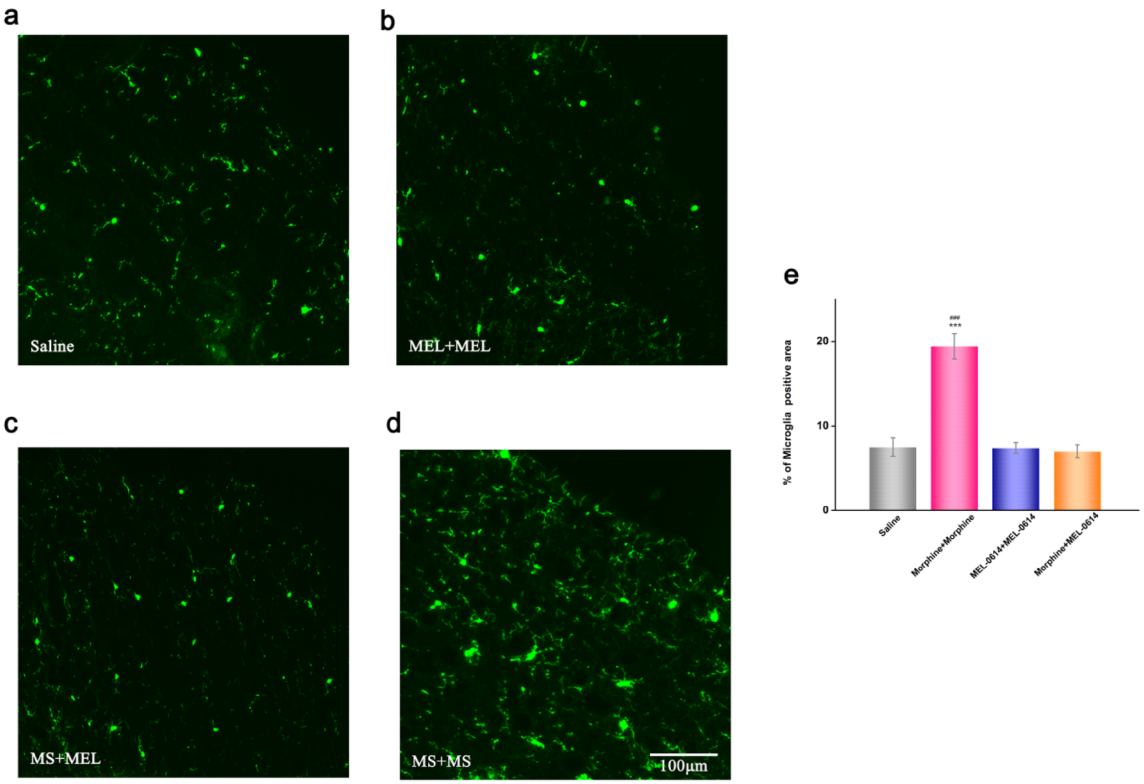

Fig. 9 The expression level of microglia in the spinal cord of SNI modle mice after IV administration of saline (**a**), MEL-0614 (**b**), morphine/MEL-0614 (**c**), and morphine (**d**), bar = 100 μm, magnification: (×10). The quantitative data describing the microglia positive areas % in the spinal cord (**e**). Data are means ± SEM of 6–12 mice. ***$P < 0.001$ indicates that response was significantly different from saline, ###$P < 0.001$ indicates that response was significantly different from MEL-0614 according to one-way ANOVA followed by Tukey's HSD test

to aggravate with continuous administration, and this phenomenon did not disappear with the withdrawal of the drug. This is consistent with the literature reports (Juni et al. 2010; Loram et al. 2012; Tumati et al. 2012). MEL-0614 did not lead to hyperalgesia until the 4th day at a high concentration (10 mg/kg), and the symptoms disappeared after discontinuation of the drug. There were no significant differences in behavioral changes between the low and medium concentrations and saline. This shows that the degree of hyperalgesia induced by mechanical stimulation was significantly lower with MEL-0614 than with morphine. Similarly, thermal hyperalgesia caused by thermal stimulation at high concentrations of MEL-0614 could be quickly recovered after the drug was stopped on day 5, but it could not be recovered with high concentrations of morphine.

The SNI model can be used to simulate the experience of common clinical peripheral neuropathic pain and is used to detect hyperalgesia induced by injected compounds in the treatment of nerve pain. Morphine reportedly has an effect on nerve pain and may aggravate it (Fletcher and Martinez 2014; Grace et al. 2016). The results also showed that IV injection of morphine can produce hyperalgesia under mechanical and thermal stimulation conditions. In contrast, under mechanical and thermal stimulation conditions, although the same dose of MEL-0614 can induce aggravation of neuralgesia, the degree of symptoms is significantly lower than that induced by morphine and they occur later. Once the drug is discontinued, the symptoms disappear. Notably, MEL-0614 showed a significant relieving effect on the neuralgesic symptoms induced by morphine, and the WT value was increased from 0.10 ± 0.01 g to 0.26 ± 0.03 g. This result may provide meaningful insights for the future clinical treatment of morphine-induced neuralgia.

Our previous study demonstrated that hyperalgesia induced by morphine and MEL-0614 could not be blocked by opioid receptor antagonists. NMDA receptors play a vital role in the production of hyperalgesia (Ma et al. 2020). Injection of traditional opioids, such as morphine, causes an increase in the expression of the NMDA receptors in the spinal cord (Gu et al. 2009; Hahnenkamp et al. 2004; Huang et al. 2019; Rivat et al. 2008). After blocking the NMDA receptors, intrathecal injection of morphine- and MEL-0614-induced hyperalgesia symptoms no longer appeared. Therefore, it is speculated that the NMDA receptors are important for pain regulation. The expression level of *Grin2b* can reflect the expression of the NMDA receptor

to a certain extent (Gong et al. 2016). The administration of morphine and MEL-0614 by IV injection may also play a role in inducing hyperalgesia. After IV morphine injection, the expression of *Grin2b* in the spinal cord of normal or SNI model mice significantly increased, but the same did not occur with MEL-0614 administration. In line with the cross-dosing behavioral results, the use of morphine combined with MEL-0614 significantly reduced the expression of *Grin2b* compared to morphine alone. These results suggest that MEL-0614 does differ from morphine in terms of NMDA receptor activation.

To further explore the reasons for the difference in hyperalgesia induced by MEL-0614 and morphine, qPCR and fluorescence experiments were used to detect the effects of IV injection of these drugs on microglia and the release of TNF and IL-1β. Pain transmission between neurons and microglia is mediated through the P2X4-BDNF-TrκB-KCC2 signaling pathway (Inoue and Tsuda 2018; Kohno and Tsuda 2021; Malcangio 2017; Wei et al. 2021). This process involves the activation of TLR4 receptors in spinal cord glial cells, upregulation of P2X4 receptors, glial cell release of BDNF, downregulation of KCC2, and finally activation of NMDA receptors to generate excitability, accompanied by the release of inflammatory mediators such as IL-1β and TNF. After 4 days of intravenous administration for normal mice or 8 days after administration for SNI model mice, the spinal cord L4–L6 segments were extracted. The qPCR results showed that in both groups, IV injection of morphine would induce an increase in the number of microglia and overexpression of inflammatory factors including TNF and IL-1β. However, the number of microglia in the spinal cord slices of mice after IV injection of MEL-0614 was not significantly different from that of the control group injected with saline, and the inflammatory mediators did not show overexpression. Especially in the SNI model, the cross-administration of morphine combined with MEL-0614 could reduce the expression of TLR4 and of other related genes to different degrees compared with the use of morphine alone. This indicates that morphine-induced hyperalgesia is related to the overexpression of these receptors and factors. Concurrently, the results could also explain the pain alleviating effect of MEL-0614 on mice in the morphine group in the behavioral experiment.

In the process of pain treatment, increasing attention has been paid to the adverse reaction of hyperalgesia. The study of hyperalgesia is very meaningful because it greatly impairs patient quality of life. In this study, we systematically explored the differences in the effects of inducing hyperalgesia under mechanical and thermal stimulation conditions after IV injection of MEL-0614 and morphine; the combined use of MEL-0614 and morphine could effectively alleviate morphine-induced hyperalgesia. At the molecular level, the degree of activation of NMDA receptors and the expression of inflammatory mediators in microglia differed after IV injection of MEL-0614 and morphine. This is of great importance for the study of the unique effects of peripheral MEL-0614 injection in neural hyperalgesia. Additionally, it potentially could be applied to alleviating morphine-induced hyperalgesia through peripheral administration. In the current study, we found that the two opioid agonists, MEL-0614 and morphine, displayed distinct roles in modulating microglia activity and development of hyperalgesia in mice, the mechanism study of MEL-0614 on regulation of pro-inflammatory cytokines and neuroinflammation should be explored in more detail in future work.

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Author Contributions RW, YW and XL designed the experiments. YW and MM wrote the manuscript. MM, JW, CH, KL, QR, and NL performed experiments. MM calculated the date and prepared the figures. All authors reviewed the manuscript.

Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no potential conflict of interests.

Ethical Approval The experiments conducted in this study met the ethical and moral requirements. To reduce the suffering of animals, each mouse was used only once. All experiments in this study were approved by the ethics committee of Lanzhou University (License Number: SYXK Gan 2009-0005).

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