

Melatonin Reduce Morphine-Induced Hyperalgesia and Tolerance of Rats via melatonin-MT1-PKC γ Pathway.

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Research

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Abstract

Background: Morphine is commonly used for treating acute and chronic pain. However, its using is complicated with tolerance and hyperalgesia. Endogenous melatonergic system is involved in development of tolerance and hyperalgesia induced by chronic morphine administration, but the precise mechanism remains unknown.

Methods: 18 male SD rats were randomly divided into Saline(Sal), Morphine(Mor) and Morphine+Chelerythrine (Mor+Che) group (n = 6, respectively). Each group were received twice-daily intrathecal injections of saline (10µl), morphine (15µg/10µl) or morphine (15µg/5µl)+ chelerythrine (5µg/5µl) for consecutive 9 days, respectively. MWT and TWL of all animals were recorded on day 1, 3, 5, 7, 9. The morphine tolerance was depicted as MPAE on day 1, 3, 5, 7, 9 and on day 10 which can be used to calculate for ED50 of each group. Then we determined the serum level of melatonin by ELISA and expression of MOR, MT1, MT2 and PKCγ by RT-qPCR and WB in [spinal dorsal horn](#) of three group rats.

Results: Comparing with Sal group, the Mor group exhibited a significant lower serum melatonin level and up-regulation of expression of the MT1, MT2 and PKCγ in the spinal dorsal horn. Co-administration of chelerythrine with morphine not only attenuates morphine-induced hyperalgesia and tolerance, but also increases the down-regulation of serum melatonin level) and reduces the up-regulation of expression of MT1 and PKCγ in spinal dorsal horn compared with Mor group.

Conclusion Melatonin can reduce morphine-induced hyperalgesia and tolerance of rats via melatonin-MT1-PKCγ pathway.

1. Background

As one of classical opioid analgesics, morphine is relied as a mainstay for acute and chronic pain management. However, the prolonged use of morphine causes hyperalgesia and tolerance[1, 2]. Morphine-induced hyperalgesia, presents as exaggerated responses to nociceptive stimulation[3]. While morphine-induced tolerance renders the patient to require increase doses of morphine to achieve target level of analgesia[4]. Although there are various overlaps in mechanism between morphine-induced hyperalgesia and tolerance[5], the precise mechanisms involved are not yet fully understood.

Melatonin, one of neuroendocrine hormones produced by pineal gland, has various important physiological effects[6, 7]. Notably, growing body of literature has shown that melatonin exerts analgesic action through MT1 and MT2, especially the latter[8]. These receptors present with high density in the superficial lamina I–V and X where play an important role in pain modulation and transmission. Moreover, there are evidences that endogenous melatonergic and opioidergic systems have crosstalk and both contribute to development of tolerance and hyperalgesia[9, 10]. Therefore, it is a promising approach to solve morphine-induced hyperalgesia and tolerance by researching their shared pathways.

Previously, we found MT1 decreased and PKC γ increased in the spinal dorsal horn of morphine-induced hyperalgesia and tolerance rats[11]. PKC γ is a main form of PKC family with crucial effects downstream of MOR[12]. It contributes to MOR desensitization which is one of main mechanisms underlying morphine-induced hyperalgesia and tolerance[13-15]. PKC can also be mediated by melatonin via MT1 and MT2[16]. Furthermore, several researches have indicated that melatonin delivery can decreased morphine-induced PKC activity or reduced expression of PKC γ [10, 17]. Thus, PKC γ may be the shared downstream of melatonin receptors and MOR. However, the definite pathway of melatonin mediating morphine-induced hyperalgesia and tolerance remains elusive. We hypothesized that melatonin reduces morphine-induced hyperalgesia and tolerance via melatonin-MT1-PKC γ pathway and we tested this hypothesis with PKC γ antagonist chelerythrine in rats with morphine-induced hyperalgesia and tolerance.

2. Methods

This study was conducted according to National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and all the procedures were done after the approval of the Institutional Animal Experimental Ethics Committee of Sichuan University (Chengdu, China, Approval NO.2017041A).

2.1 Experimental animals

Adult male Sprague-Dawley rats weighing 230-300 g (n = 18) provided by Chengdu Dossy Biological Technology Co., Ltd (Chengdu, China) were housed in separate cage with food and water freely. The room has 24°C maintained temperature with a 12-hour light/darkness cycle. All experiments were performed during 8 am to 4 pm on each test day and all behavioral assessments were performed in the morning.

2.2 Intrathecal catheter implantation

The procedure of intrathecal (i.t.) catheter insertion was carried out as described in our pervious study[11]. In brief, pentobarbital sodium (60 mg/kg, i.p.) was used to anesthetize rats. Then cervical and lumbar dorsal skin was sterilized with povidone-iodine. An incision about 1.5cm on the middle line of lumbar skin (L4–S1) was made. The bilateral muscle along L5 spinous process was cut. Then the L5 spinous process was removed and the intervertebral membrane between L4 and L5 was exposed. The ligamentum flavum was then punctured by a 22gauge needle following a sterile polyethylene (PE-10) catheter was inserted into the subarachnoid space rostrally about 2cm length. The catheter was fixed to the muscle and anadesma, and the proximal ends of the catheter were tunneled underneath the skin and exist out to the cervical region. After injecting 5 μ l saline to ensure patency of the catheter, the tip of the catheter was sealed via fusing. The wounds were sutured layer by layer by 5-0 surgical sutures. The animals were allowed to recover for 3 days in individualized cages before use. The correct placement of the catheter was then verified by observing symmetrical lower limbs paralysis induced by intrathecal injection of 10 μ l 2% lidocaine and completely recovered from the spinal anesthesia on the fourth day

after surgery. The animals showing any signs of prolonged motor impairment after the spinal anesthesia were excluded. The correct placement of the catheter in the subarachnoid space at the lumbar enlargement was confirmed by dissection at the end of experiment.

2.3 Drug and vehicle administration

Morphine hydrochloride (Shenyang First Pharmaceutical Factory, Shenyang, China) was dissolved in saline. Chelerythrine was dissolved in Dimethyl Sulfoxide (DMSO). Eighteen rats were divided randomly into three groups, Saline (Sal), Morphine (Mor) and Morphine+ Chelerythrine (Mor+Che) (n=6, each). The Mor group received twice-daily i.t injections of morphine (15µg/10µl) followed by a flush of 10µl saline delivery into the subarachnoid space for consecutive 9 days—twice a day at 9AM and 4PM, respectively) using a microinjection syringe (Gaoge, Shanghai, China). The Mor+Che group received twice-daily i.t injections of morphine (15µg/5µl) and chelerythrine (5µg/5µl) followed the same flush as Mor group. The Sal group received the same amount of 20µl saline, but no morphine, in the same fashion as done for the morphine group.

2.4 Behavioral assessments

Prior to the first behavioral test, rats were adapted to the testing environment for 3 h per day for consecutive 5 days. Habituation covers the process of moving rats from feeding room to the testing room and then they were kept in the testing equipment for 3 h. The behavioral test was implemented by a researcher blinded to the rat groups. Mechanical hindpaw withdrawal threshold and thermal withdrawal latency of all animals were recorded on the 1, 3, 5, 7, 9 day respectively during drugs injection. The baseline nociceptive thresholds (mechanical withdrawal threshold followed by thermal withdrawal latency) on right hindpaw of each group were determined prior to drugs administration on each designated test day. The thermal withdrawal latency was measured again 30min after the first drug or vehicle administration per day between 9:00 am to 10:00 am. After the final behavioral test, rats were euthanatized by anesthetization with pentobarbital sodium (60 mg/kg, i.p.) followed by cervical dislocation and the L3-L5 spinal cord were harvested.

2.4.1 Mechanical allodynia

Mechanical withdrawal threshold (MWT) was measured via a von Frey filament marked with a calibrated range of bending force (0.6, 1, 1.4, 2, 4, 6, 8, 10, 15, and 26 g) as described previously. Each rat was put into a plastic cage having a wire mesh bottom and allowed to acclimate for 30 min before measurement. A single von Frey filament was applied vertically to the region between feet pads in the aspect of right hind paw plantar for five times with an interval of 5 s. At least one distinct withdrawal response out of five applications was treated as a positive record. Filaments were used in an up-and-down rule in accordance with a negative or positive response to determine the hindpaw withdrawal threshold.

2.4.2 Thermal hyperalgesia

Thermal withdrawal latency (TWL) to radiant heat was determined according to a previously described method using a thermal testing apparatus (Ugo Basile, Plantar Test 37370, Italy). Rats were placed individually into plexiglas cubicles placed on a transparent glass surface and allowed to acclimate for 30 min before testing. The light beam from a projection bulb which was located below the glass was directed at the region between feet pads in the plantar aspect of right hindpaw. The period from the beginning of radiant heat stimulation to withdrawal of the hindpaw was regarded as hindpaw withdrawal latency. The baseline latency of about 12 s and a cut-off time of 20 s which was set to prevent thermal injury. Three trials with an interval of 5 min were made and scores from three trials were averaged to yield mean withdrawal latency. The measurements were implemented both before and 30 min after morphine administration.

2.4.3 Morphine tolerance

The morphine tolerance was depicted as the percentage of maximal possible antinociceptive effect (%MPAE) in the morning from day 1 to 9 and on day 10. The %MPAE was calculated as $\%MPAE = [(Test\ PWL - Basic\ PWL) / (cutoff\ time - Basic\ PWL)] \times 100$. Cumulative dose-response curves were determined on day 10 after long-term morphine and saline administration according to previously described method[11]. Rats were firstly administrated with a low dose morphine (10 μ g) followed by antinociceptive effect assessment 30 minutes later through TWL test and then received a next dose (cumulative dosing increase by 2-fold) and were tested again 30 minutes afterward until the rats couldn't feel the stimulation so that failed to move the hindpaw within the cutoff time.

2.5 ELISA

On day 10, all rats were deeply anesthetized with pentobarbital (60 mg/kg, i.p.). The blood was taken from ventriculus dexter in the afternoon to minimize fluctuation of the serum melatonin level. The blood was centrifuged at 1500rpm for 15min and the serum was obtained and placed in EP tubes wrapped with aluminum foil for protection from light and stored at -80 °C until use. The level of serum melatonin was assayed though a rat-specific ELISA kits (XL-Er1047, YonghuiBiological Technology Co., Ltd, Beijing) according to the manufacturer's instructions.

2.6 Real-Time (RT)-PCR

A part of tissues harvested from each rat for preparation of western blot were used for RT-PCR. Firstly, the tissues were isolated for the total RNA extraction. Single-strand complementary DNA was designed using Primer Premier 6.0 and synthesized by Sangong Biotech (Shanghai,China). All complementary DNA samples were stored at -80°C before using. RT-PCR was performed using a PikoReal Real-Time PCR

System (PIKORed 96, ThermoFisher, US). Relative messenger RNA (mRNA) levels were calculated according to the 2- $\Delta\Delta$ CT method. Oligonucleotide primers were used as Table1.

Table 1 Sequences of primers for RT-PCR		
Target gene	Forward primers	Reverse primers
β -actin	gaagatcaagatcattgctcct	tactcctgcttgctgatcca
MT1	tgtcgcttacaccgttgctccagat	ggcagcagcactgttcgtcacttca
MT2	tgatctccaactgccgcctccattc	agctgcacttggtccaaagcctctt
MOR	tctcttctgccatcggtctgcctgt	tacagccacgaccaccagcaccat
PKC γ	ggttcaccgccgatgccacgaatt	ctctgtatgggtccacgccgcaaagg

2.7 Western blot

Pentobarbital (60 mg/kg, i.p.) was used to anesthetize rats in each group (n = 3/6) and decapitation was performed for rapid tissue harvesting. The L3-L5 segment of the spinal cord was first divided into the right and left side and then further separated into the dorsal and ventral horn, respectively. Tissue samples were homogenized in RIPA buffer (50mM Tris (pH 7.4),150mM NaCl,1% NP-40,0.1% sodium deoxycholate) and were allowed to homogenize for 10min on fragmentized ice. The samples were centrifuged at 4°C for 15 min at 12000rpm. The protein concentration of the supernatants was assayed via a BCA protein assay kit (KGP903, KenGEN BioTECH, Jiangsu, China). Supernatants (50 μ g) were warmed for 5 min at 100°C in loading buffer (50 mM Tris–HCl, 2 %SDS, 2 % β -mercaptoethanol, 8 % glycerol, and 0.1 % bromophenol blue) and resolved by SDS-PAGE on 10 % polyacrylamide gels. After gel electrophoresis, proteins were electrophoretically transferred to PVDF membranes (HYBOND, Inc.USA). The membranes were blocked via 5%BSA in Tris-buffered saline (TBST) with 0.1 % Tween 20 for 2 h and incubated overnight at 4 °C with a primary antibody to MOR(1:200, ab10275, Abcam, Cambridge, UK), MT1 (1:500, NBP1-71113, Novus Biologicals, US), MT2 (1:200, ab203346, Abcam, Cambridge, UK), PKC γ (1:200,ab71558, Abcam, Cambridge, UK), β -actin (1:5,000) Membranes were then incubated in HRP conjugated secondary antibody (1:5,000; ab6721 and ab6789, Abcam, Cambridge, UK) for 2-3 h at room temperature, visualized with TBST for 3 times (10min per time), and exposed onto X-films for 1–5 min. The X-films were scanned and the density of target bands was analyzed by Image Lab (BIO-RAD, US)

2.8 Statistical data analysis

Data are presented as mean \pm SEM. The SPSS 16.0 (Chicago, IL) was used to conduct statistical analysis and the figures were presented by GraphPad Prism 6.0 software (La Jolla, CA, USA). Alteration of MWT, TWL and MPAE% of day1 to day 9 between groups were assessed with two-way analysis of variance

(ANOVA) group \times day followed by the Bonferroni post hoc test for multiple comparisons. The data of ELISA, western blot, RT-PCR of each group were compared by one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test for multiple comparisons. To examine the degree of morphine tolerance, the ED₅₀ value of morphine in each group was determined via the formula calculated from linear curve estimation when $y=0.5$. The up-down reversals were used to get ED₅₀ dose and 95% confidence intervals (95% CI) of morphine anti-nociception effect and the probit analysis was applied for the three groups. P-values <0.05 was considered statistically significant.

3. Results

3.1 Effect of co-administration of chelerythrine with morphine on development of hyperalgesia.

The mechanical withdraw threshold (MWT) of Sal group remained unchanged over a period of 9 days. Administration of morphine alone or morphine plus chelerythrine lead MWT go down significantly starting from day 3 to the last test day. The MWT reduction tendency of Mor group moderated through test day 7 and day 9 whereas the MWT reduction tendency of Mor+Che group moderated through test day 5 to day 9. Comparing with that on day 1, MWT of Mor group was reduced by 96.10% on the day 9. On the other hand, MWT of Mor+Che group was only reduced by 60.16% on the day 9 comparing with that on day 1 (Figure 1A). Meanwhile, thermal withdraw latency (TWL) showed almost no change during 9 days of course in the Sal group. TWL in Mor group showed significant reduction on day 3 and continued to decrease and it seemed reaching its steady state on day 9. On day 9, it was reduced by 65.41%. On the other hand, TWL in Mor+Che group also showed significant reduction on day 3 but it seemed reaching its steady state on day 5. On day 9, it was only reduced by 40.48%. (Figure 1B).

3.2 Effect of co-administration of chelerythrine with morphine on development of morphine tolerance

Our data show long-term intrathecal morphine delivery drives dose escalation of morphine to get maximal possible antinociceptive effect (MPAE), but co-administration of chelerythrine with morphine mitigates that effect (Figure 2A). MPAE of Mor group from day 3, 5, 7, 9 decreased 43.79% ($p < 0.001$), 79.01% ($p < 0.001$), 91.82% ($p < 0.001$), 97.04% ($p < 0.001$), whereas MPAE of Mor+Che group from day 3, 5, 7, 9 decreased 13.22% ($p > 0.05$), 20.53% ($p < 0.001$), 32.43% ($p < 0.001$), 44.70% ($p < 0.001$), compared with the value on day 1 respectively. Chronic intrathecal morphine or co-administration of chelerythrine with morphine both produced a rightward shift in the cumulative dose response curve of morphine on day 10, but the rightward shift level of Mor+Che group is less than that of Mor group (Figure 2B). The morphine ED₅₀ values of rats in three groups on day 10 were presented in Table 2. The ED₅₀ for morphine, the morphine dose needs to produce 50% of the maximal anti-nociceptive effect of morphine, was 6.44 μ g, 26.04 μ g, 15.30 μ g for Sal, Mor and Mor+Che group respectively. ED₅₀ of Mor group was 4.03 times of that of Sal group and 1.70 times of that of Mor+Che group on day 10 ($p < 0.001$). (Table 2)

Table 2. ED50 and 95% CI of morphine in Sal, Mor and Mor+Che group

	ED ₅₀ (μg)	95% CI (μg)
Sal group	6.44	4.82 to 7.16
Mor group	26.04	23.29 to 28.30***
Mor+Che group	15.30	13.53 to 17.06***###
The measurement was conducted on day 10 after completion of morphine delivery procedures for 9 consecutive days. ED ₅₀ : the morphine dose that produced 50% of the maximal possible anti-nociception effect 95% CI: 95% confidence intervals. *** $p < 0.001$, as compared with the Sal group on day 10, ### $p < 0.001$, as compared with the Mor group on day 10.		

3.3 Effect of co-administration of chelerythrine with morphine on the serum melatonin level of rats with morphine-induced hyperalgesia and tolerance.

The level of serum melatonin was determined on day 10. The serum melatonin level in Mor was lower than that of Sal group ($p=0.0036$). Whereas the serum melatonin level in Mor+Che group was lower than Sal group ($p=0.0425$) but higher than Mor group ($p=0.0425$) (152.63 ± 6.94 for the Sal group vs. 92 ± 4.86 for Mor group vs. 120.03 ± 9.78 for Mor+Che group). (Figure 3).

3.4 Effects of chronic intrathecal co-administration of chelerythrine with morphine on expression of MOR, MT1, MT2 and PKC γ in the spinal cord dorsal horn of rats with morphine-induced hyperalgesia and tolerance.

After intrathecal delivery of saline, morphine or morphine+ chelerythrine for 9 consecutive days, we measured mRNA of MOR, MT1, MT2 and PKC γ in rat's spinal dorsal horn on day 10. The mRNA of MT1, MT2, PKC γ in Mor group were all higher than those of Sal group ($p=0.0014$, $p=0.027$, $p=0.0008$, respectively). There was no significant difference in mRNA levels of MOR between the Sal group, Mor group and Mor+Che group ($p>0.05$). (Figure 4). The mRNA of MT1, PKC γ in Mor+Che group were lower than those of Mor group ($p=0.006$, $p=0.0005$, respectively), but no significant difference in mRNA level of MT2 was shown between the Mor group and Mor+Che group ($p>0.05$). Beside that mRNA level of MT2 in Mor+Che group was higher than that of Sal group ($p=0.0301$), there was no significant difference in mRNA levels of MOR, MT1 and PKC γ between the two groups ($p>0.05$) (Figure 4).

Protein level of MOR, MT1, MT2 and PKC γ were determined using Western blot analysis. In Mor group, the protein level of MT1, MT2, PKC γ were higher than those in Sal group ($p=0.0001$, $p=0.0108$, $p=0.0001$, respectively). While the protein of MOR in Mor group showed no significant difference with that in Sal group ($p>0.05$). There was no significant difference in protein level of MOR and MT2 between the Mor group and Mor+Che group ($p>0.05$). While the protein of MT1 and PKC γ protein in Mor+Che group reduced significantly compared with those in Mor group ($p=0.0005$, $p<0.0001$, respectively). The MT2 protein of Mor+Che group increased significantly ($p=0.0078$) while the PKC γ reduced significantly compared with

Sal group ($p=0.0002$). The MOR and MT1 protein of Mor+Che group showed no significant difference with those in Sal group ($p>0.05$). (Figure 5).

4. Discussion

In the present study, we found that intrathecal morphine for 9 consecutive days leads to morphine-induced hyperalgesia and tolerance in adult rats, and reduces the serum melatonin level, increased melatonin receptor MT1, MT2 and PKC γ in spinal dorsal horn of rats. Co-administration of chelerythrine with morphine not only reduces morphine-induced hyperalgesia and tolerance, but also increases the reduced serum melatonin level and down-regulates the higher level of MT1 and PKC γ . But no significant change of MT2 in spinal dorsal horn of rats was observed by co-administration with chelerythrine.

4.1 PKC γ plays a crucial role in development of morphine-induced hyperalgesia and tolerance

As our previous study[11], intrathecal administering morphine to rats for 5 consecutive days can result in behavior of hyperalgesia and tolerance. But in that study, the degree of morphine hyperalgesia and tolerance is not severe enough and the state has not stabilized. Because the rate and extent of development of morphine tolerance or hyperalgesia is related to the amount of morphine used and the duration of administration[18]. It is helpful to observe changes of the indicators to be detected when the model is in a steady state. Therefore, we not only prolonged the duration of medication for 9 days, but also increased the dosage to 30 μ g per day in our current study compared to 5 days and 20 μ g per day of previous study. Our results show that, compared with Sal group, the force causing equal level of pain and TWL reduced in Mor group. The tendency of MWT and TWL of Mor group moderated through test day 7 and day 9 seeming to have reached severe enough hyperalgesia and a stable state. Co-administration of chelerythrine with morphine minimized morphine-induced hyperalgesia and tolerance. These results suggest PKC γ inhibitor chelerythrine can alleviate morphine-induced hyperalgesia and tolerance effectively.

Several studies have reported the activation of PKC γ in the spinal dorsal horn plays an important role in the development of morphine-induced hyperalgesia and tolerance[14, 15], and this effect may act through mediating MOR phosphorylation[12]. There is no analgesic effect of chelerythrine itself[10]. Our results show that PKC inhibitor chelerythrine can significantly reduce morphine-induced hyperalgesia and tolerance, which is consistent with previous studies [14, 15, 19].

4.2 Melatonergic system participates in morphine-induced hyperalgesia and tolerance at multiple levels.

Our result showed decreased serum melatonin level in rats with morphine-induced hyperalgesia and tolerance, which is consistent with our previous study[11]. Shavali et al[20] found that melatonin exerted analgesic effect not through opioid receptors but via causing the release of beta-endorphin. Furthermore, analgesic effect of morphine decreases after excision of the pineal gland where is the main place secreting melatonin[21]. Studies have shown that melatonin can regulate pain through the endogenous pain-modulating system (PMS)[22]. Administration of exogenous melatonin can alleviate allodynia and

hyperalgesia in neuropathic pain models[23], and this analgesic effect is dose-dependent[24, 25]. This indicates the decrease of melatonin level is associated with decreased MWT and TWL in rats. Thus, to a certain extent, reduction in serum melatonin level is involved in morphine-induced hyperalgesia and tolerance. But we didn't point out the precise location it works. It can happen either at peripheral or central or both.

Melatonin receptors also involve in morphine-induced hyperalgesia and tolerance. Our study demonstrated increased MT1 and MT2 level in rats' spinal dorsal horn with morphine-induced hyperalgesia and tolerance which is different with our pervious study that administration of morphine lead MT1 down-regulation but no change in MT2[11]. The development of tolerance and hyperalgesia can be affected by both duration and dose of morphine, because the varying of both pharmacokinetic and pharmacodynamic factors [18]. We therefore speculate the difference may due to the prolonged duration of morphine administration or the increased dose of morphine administered. It is suggested that the melatonin receptors' level in the rats' spinal dorsal horn of may have a dynamic fluctuation process at different stages in development morphine hyperalgesia and tolerance. Meanwhile, we speculate that this volatility change in MT1 is characterized by a decrease first and then an increase. Niles [26] and Bahna[27] et al. found that MT1 and MT2 were up-regulated in rats' hippocampus after valproic acid induced neuropathic pain stimulation, suggesting that melatonin receptors are dynamically regulated by remodeling of pain-related nervous systems[23], which support our speculation. Furthermore, it suggests the MT1 and MT2 up-regulation is a feedback adjustment consequence to pain-related nervous system change due to morphine hyperalgesia and tolerance. Since the rat spinal dorsal MT2 level was not measured in the first five days of the study, it is not clear whether the MT2 level also reduced first and then increased as MT1 or directly increased.

4.3 Endogenous melatonergic system involves in development of chronic morphine-induced hyperalgesia and tolerance through serum melatonin-MT1-PKC γ pathway

PKC γ , especially which in the spinal dorsal horn, plays an important role in development of morphine-induced hyperalgesia and tolerance[28, 29]. In the present study hyperalgesia and tolerance was observed with increased PKC γ in rats' spinal dorsal horn, and was ameliorated by PKC antagonist treatment. This consist with previous studies demonstrated morphine hyperalgesia and analgesic tolerance can be attenuated by co-administration of morphine with inhibitors of PKC effectively[29, 30]. In addition, Wei et al[17] and Li et al[10] found that administration of exogenous melatonin can alleviate morphine-induced hyperalgesia or tolerance by inhibiting PKC γ activation or reducing the up-regulation of PKC γ expression. Our study showed blocking PKC γ increased the down-regulated serum melatonin level and reduced morphine-induced hyperalgesia and tolerance, suggesting PKC γ also involves in the mechanism underlying the effect of melatonin mitigates morphine-induced hyperalgesia and tolerance. Melatonin exerts its' antinociceptive effects mainly through MT1 and MT2[25], and pervious report has demonstrated melatonin can activate PKC via its' receptor MT1/ MT2[16]. However, we found blocking PKC γ only decreased MT1 but not MT2, indicating that melatonin attenuated morphine-induced hyperalgesia and tolerance probably via serum melatonin-MT1-PKC γ pathway.

5. Conclusion

Our study demonstrated that melatonin is involved in hyperalgesia and tolerance induced with long-term administering morphine and PKC γ antagonist chelerythrine minimize the severity of hyperalgesia and tolerance. It seems that melatonin can reduce morphine-induced hyperalgesia and tolerance of rats via melatonin-MT1-PKC γ pathway.

List Of Abbreviations

PKC: protein kinase C; MOR: μ -opioid receptor; MT: melatonin receptor; Sal: Saline; Mor: Morphine; Che: chelerythrine; mechanical withdraw threshold (MWT); thermal withdraw latency (TWL); maximal possible antinociceptive effect (MPAE)

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Figures

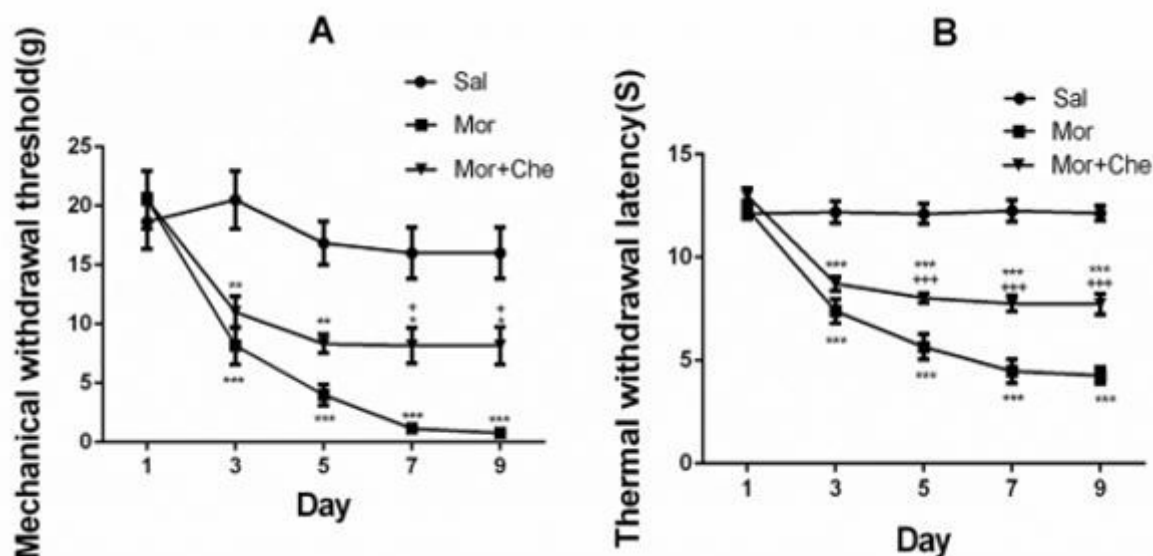


Figure 1

Effect of long-term intrathecal co-administration of chelerythrine with morphine on mechanical withdraw threshold and thermal withdraw latency (A) The mechanical withdrawal threshold (MWT) of rats. Comparing with Sal group, MWT of Mor group was reduced by 61.16%, 76.24%, 92.71%, 95.00% on day 3, 5, 7, 9 respectively and was reduced by 46.34%, 50.50%, 48.96%, 48.96% on day 3, 5, 7, 9 respectively in Mor+Che group. (B) The thermal withdrawal latency (TWL) of rats. Comparing with Sal group, TWL of Mor group was reduced by 39.38%, 53.25%, 63.46%, 64.85% on day 3, 5, 7, 9 respectively and was reduced by 28.49%, 33.72%, 36.67%, 36.34% on day 3, 5, 7, 9 respectively in Mor+Che group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with Sal group on the corresponding days and analyzed by Bonferroni correction; + $p < 0.05$, +++ $p < 0.001$, compared with Mor group on the corresponding days and analyzed by Bonferroni correction; + $p < 0.05$, +++ $p < 0.001$. Data is presented as mean \pm SEM, $n=6$ in each group.

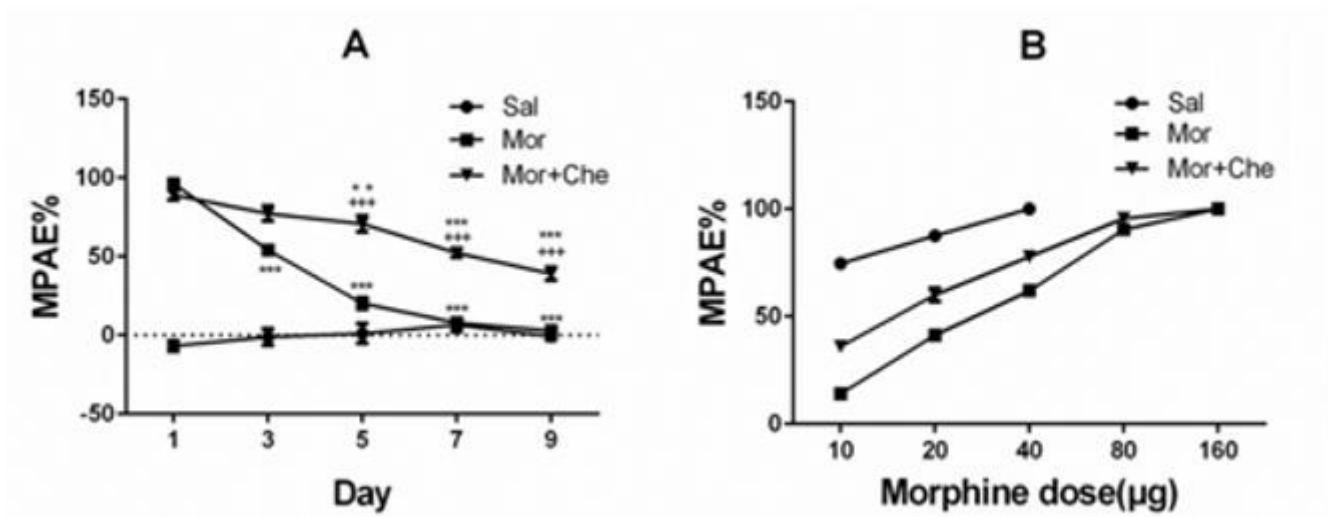


Figure 2

Effect of chronic intrathecal co-administration of chelerythrine with morphine on MPAE (A) MPAE vs. number of day of administering saline, morphine and morphine+ chelerythrine, ** $p < 0.01$, *** $p < 0.001$, compared with the value on day 1; ++ $p < 0.05$, +++ $p < 0.001$, compared with the value on the same day in Mor group. (B) Morphine cumulative dose response curve of Sal group, Mor group and Mor+Che group. After completion of consecutive 9 days of intrathecal solution delivery, the cumulative dose response curve of morphine to achieve MPEA was generated on day 10. Data are presented as mean \pm SEM, $n = 6$ rats for each group.

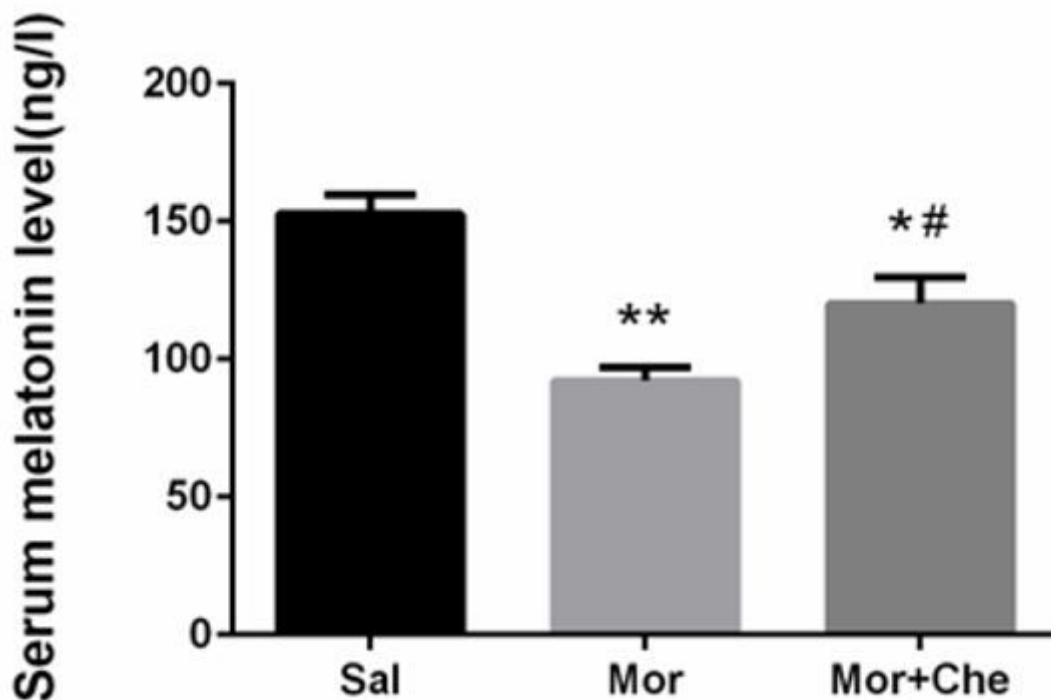


Figure 3

Serum melatonin level of the rats after receiving saline, morphine or morphine+chelerythrine administration for 9 consecutive days The serum melatonin was measured on day10 using ELISA, * $p < 0.05$, ** $p < 0.01$, compared with Sal group; # $p < 0.05$, compared with Mor group. Data is presented as mean \pm SEM, $n=6$ for each group.

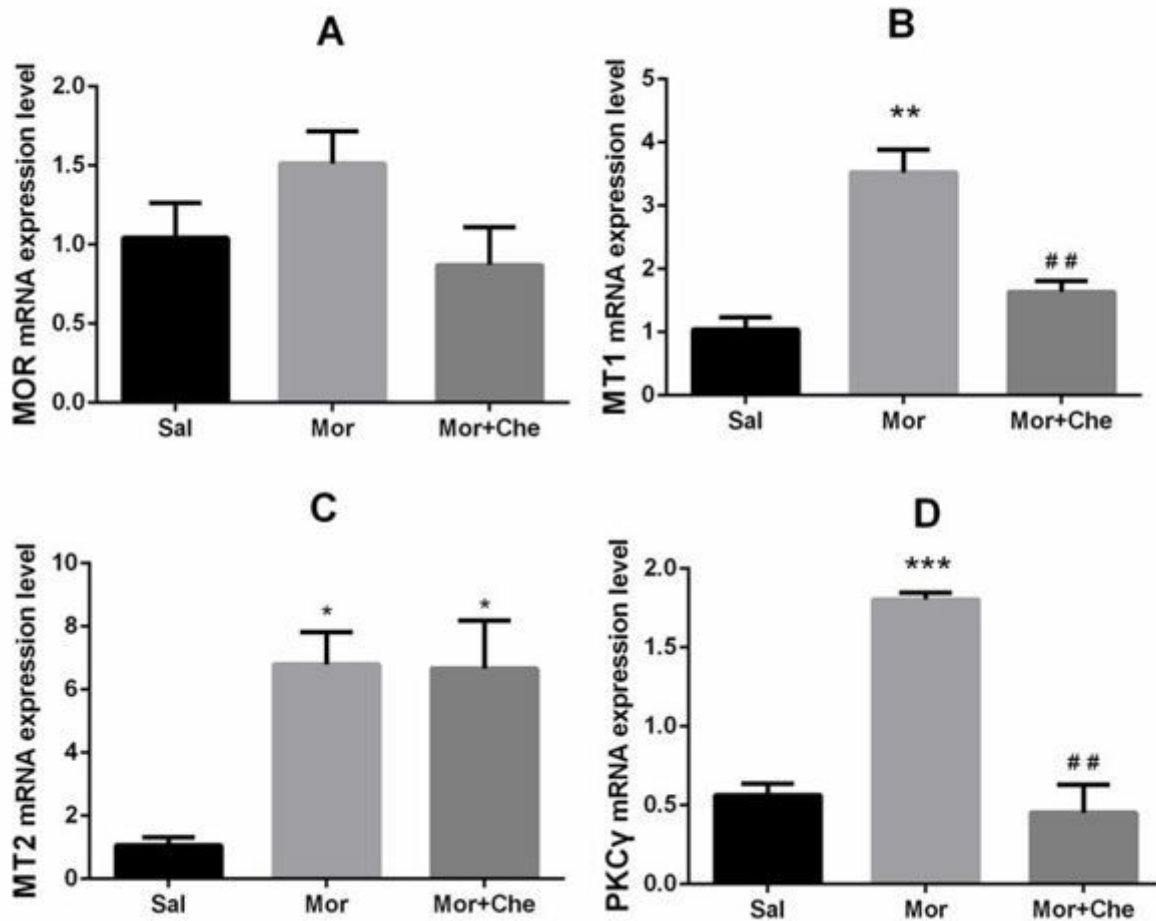


Figure 4

mRNA level of MOR, MT1, MT2, and PKCγ in the spinal dorsal horn of the rats in Sal, Mor and Mor+Che group (A) There was no significant difference in mRNA level of MOR between Sal, Mor and Mor+Che group on day 10 after delivery of drug or vehicle for 9 consecutive days. (B) The mRNA expression of MT1 in Mor group was significantly higher than that in Sal group, and it in Mor+Che group was significantly lower than that in Mor group. (C) The mRNA of MT2 of Mor and Mor+Che group were significantly higher than that in Sal group. (D) PKCγ mRNA level was significant higher in Mor group than that of Sal group, and it in Mor+Che group was significantly lower than that in Mor group. * $p < 0.05$, *** $p < 0.001$, compared with Sal group; # $p < 0.05$, ## $p < 0.01$, compared with Mor group. Data are presented as mean \pm SEM, $n= 3$ for each group.

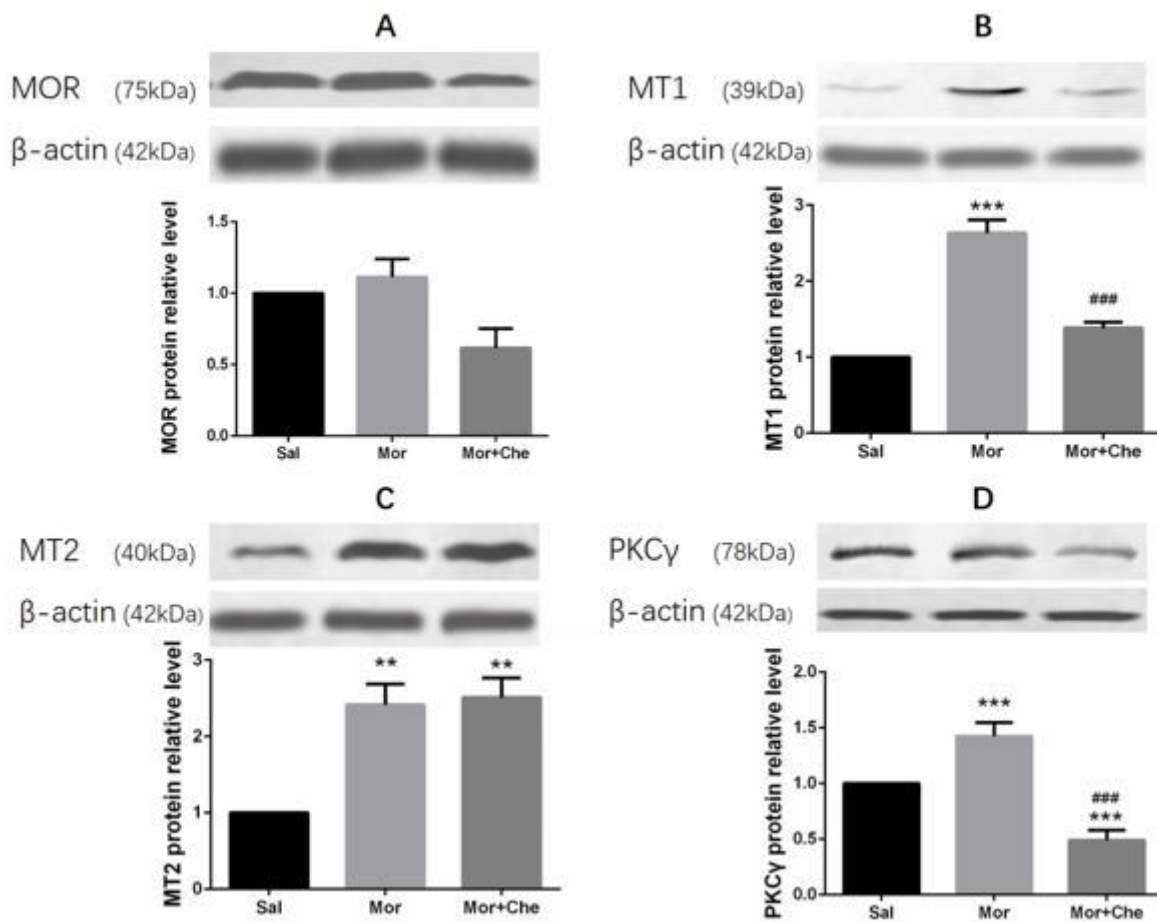


Figure 5

Protein level of MOR, MT1, MT2 and PKC γ in the spinal dorsal horn of rats in Sal, Mor and Mor+Che group. Protein levels were measured with Western blot with on day 10 after intrathecal delivery of drug or vehicle for 9 consecutive days. (A) There was no significant difference in MOR protein levels between three groups. (B) MT1 protein level in Mor group was significantly higher than that of Sal group, and it in Mor+Che group was significantly lower than that in Mor group. (C) The MT2 protein levels in Mor and Mor+Che group were significantly higher than that of Sal group. (D) PKC γ protein level of Mor group was significantly higher than that of the Sal group, and it in Mor+Che group was significantly lower than that in Mor group. PKC γ protein level of Mor+Che group was significantly lower than that of the Sal group. ** $p < 0.01$, *** $p < 0.001$, compared with Sal group; ### $p < 0.001$, compared with Mor group. Data are presented as mean \pm SEM, $n = 3$ rats for each group.

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