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ORIGINAL PAPER

Systemic Rapamycin Attenuates Morphine-Induced Analgesic Tolerance and Hyperalgesia in Mice

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Abstract

Previous studies showed that repeated intrathecal morphine injection activated the mammalian target of rapamycin complex 1 (mTORC1) in spinal dorsal horn neurons and that blocking this activation by intrathecal infusion of rapamycin, a specific mTORC1 inhibitor, prevented the initiation of morphine-induced tolerance and hyperalgesia. However, in clinic, rapamycin is usually administrated orally. In this study, we examined whether systemic administration of rapamycin had the effect on morphine-induced tolerance and hyperalgesia in mice. Repeatedly intraperitoneal injection of morphine led to morphine analgesic tolerance on day 5 post-injection evidenced by a marked decrease in morphine's maximal possible analgesic effect and hyperalgesia on day 6 post-injection demonstrated by significant increases in paw withdrawal frequency in response to mechanical stimulation and decreases in paw withdrawal latency in response to cold stimulation on bilateral sides. Co-intraperitoneal injection with rapamycin prevented the development of morphine analgesic tolerance and hyperalgesia. Moreover, on day 6 after morphine injection, co-intraperitoneal injection with rapamycin reduced the established morphine tolerance and hyperalgesia. Co-intraperitoneal injection of rapamycin also attenuated the morphine-induced increases in the levels of phosphorylated mTOR and its downstream target phosphorylated 4E-BP1 in the spinal cord dorsal horn. Our findings indicate that, like intrathecal injection, systemic administration of rapamycin has significant effects on both induction and maintenance of morphine tolerance and hyperalgesia. Systemic mTOR inhibitors could serve as promising medications for use as adjuvants with opioids in clinical chronic pain management.

Keywords mTORC1 · Morphine tolerance · Morphine hyperalgesia · Systemic administration

Introduction

Opioids such as morphine are still the gold standard treatment for acute and chronic pain [1].However, long-term use of morphine often produces severe side effects, such as analgesic tolerance and hyperalgesia [2]. Morphine analgesic tolerance is characterized by a reduced response to the analgesic effects of morphine, which require a higher dose to achieve equivalent pain relief [2], while morphine-induced hyperalgesia is a neuronal sensitization process in which morphine paradoxically produces pain hypersensitivity, which can be manifested as thermal hyperalgesia, mechanical and cold allodynia [2].

The neurobiological mechanisms of opioid tolerance and hyperalgesia after chronic opioid exposure include [1] phosphorylation, desensitization, internalization and down-regulation of opioid receptors [3] or heterodimerization with other receptors leading to an enhanced perception of pain [2, 4] enhanced excitatory primary afferents, descending spinal facilitation, glial cell activation, and up-regulation of dorsal horn intracellular nNOS, PKCγ, and CaMKII signaling [5]. The intracellular processes caused by the above changes may initiate the translation of multiple proteins, contributing to the mechanism of opioid-induced tolerance and hyperalgesia [6]. The mammalian target of rapamycin (mTOR) is a serine-threonine protein kinase, which is generally known to gate translation of most proteins by phosphorylation of specific downstream

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effectors, such as p70 ribosomal S6 protein kinases (S6Ks) and the eukaryotic initiation factor 4E-binding (eIF4E-binding) proteins (4E-BPs) [7, 8]. Once mTOR binds to Raptor protein, it forms mTOR complex1 (mTORC1) and phosphorylates downstream effectors [7, 8]. Previous work has demonstrated that spinal mTORC1 plays a key role in the initiation and maintenance of morphine-induced tolerance and hyperalgesia [6, 9]. Repeated intrathecal infusion of morphine activated mTOR, p70S6K and 4E-BP1 in rat spinal dorsal horn neurons [6, 9]. Cellular membrane μ opioid receptor triggered this activation through intracellular PI3K/Akt signal pathway in dorsal horn neurons [6]. Intrathecal administration of rapamycin, a specific and selective inhibitor of mTOR activity in mTORC1 attenuated both the induction and maintenance of morphine tolerance and hyperalgesia [6]. These effects were attributed to the attenuation of morphine-induced increases in translation initiation activity, nascent protein synthesis, and the expression of some known key tolerance-associated proteins, such as neuronal NOS (nNOS), in the dorsal horn [6, 10]. Further study on neuropathic pain rats showed that intrathecal infusion of rapamycin delayed morphine-induced tolerance and relieved morphine-induced hyperalgesia under the fifth lumbar spinal nerve ligation (SNL)-induced neuropathic pain conditions [9]. However, all of these experiments carried out intrathecal infusion/injection of rapamycin. Given that systemic drug administration is most popular and convenient in clinic and that the mTOR inhibitors currently are given orally in clinic [11], the goal of current study was to examine whether systemic administration of rapamycin had the effect on the initiation and maintenance of morphine-induced tolerance and hyperalgesia.

Materials and Methods

Animals and Drugs

Male C57BL6 weighing 25–30 g were housed with food and water ad libitum on a 12 h light/12 h dark cycle. Animals were exposed to habituation for 2 days prior to use. Animal protocols are approved by the Institutional Animal Ethics Committee of Nankai University. All animal experimental procedures follow ethical guidelines produced by the Institutional Animal Ethics Committee of the International Association for the Study of Pain. Every effort was made to minimize the amount of animals used and their suffering. Behavioral tests were completed in a blinded manner.

Morphine (NEGPF, China), rapamycin (Sigma-Aldrich, St. Louis, MO), and ascomycin (MedChemExpress, NJ) were used in this study.

Behavioral Testing

Tail flick test was carried out in mice as described previously [12]. In brief, tail flick latency to noxious heat was measured on day 0 (prior to morphine injection), and days 1, 3, 5, 8 and 10 using an Analgesic Meter (Model 33B Tail Flick Analgesia Meter, IITC Life Science, Woodland Hills, CA, USA). The cut-off time was 10 s. Three trials were conducted for each rat with an interval of 3 min. Morphine's maximal possible analgesic effect (MPAE) was calculated using the following equation:

$$\mathrm{MPAE} = \left[(\mathrm{Posttreatment} - \mathrm{Pretreatment})/(10 - \mathrm{Pretreatment})\right] \times 100\%.$$

Cold test was used to examine paw withdrawal latency (PWL) to noxious cold (0 °C) as described previously [13]. The animal was placed in an individual Plexiglas chamber on the cold aluminum plate, the temperature of which was monitored continuously by a thermometer. The PWL was recorded by the length of time between the placement of the hind paw on the plate and a flinching of the paw. Each test was repeated three times at 10-min intervals for the paw on the ipsilateral side. To avoid tissue damage, a cut-off time of 20 s for mice was used.

Mechanical test was carried out as described [12]. Briefly, each mouse was placed in a Plexiglas chamber on an elevated mesh screen. One calibrated von Frey filament (0.07 g; Stoelting Co., WoodDale, IL, USA) was applied to the hind paw for approximately 1 s, and each stimulation was repeated 10 times to both hind paws. The occurrence of paw withdrawal in each of these 10 trials, indicated as paw withdrawal frequency (PWF), was expressed as a percent response frequency [(number of paw withdrawals/10 trials) × 100%].

Morphine Analgesic Tolerance, Morphine-Induced Pain Hypersensitivities and Drug Co-administration Procedure

For morphine analgesic tolerance development period, mice received subcutaneous (s.c.) injection of 20 mg/kg morphine (NEGPF) twice a day and intraperitoneal injection of 2.5 mg/kg rapamycin (Sigma-Aldrich) or vehicle (50% DMSO) once a day for 5 days. The tail flick test was performed before morphine injection and at 0.5 h after s.c. injection of an inducing dose of morphine (10 mg/kg) in naive mice on mornings 1, 3, and 5 in naive mice. The cold and mechanical tests were performed before morphine injection and on day 6 after first morphine injection.

For morphine analgesic tolerance maintenance period, mice received subcutaneous injection of 20 mg/kg twice a

day for 10 days and also intraperitoneal injection of 2.5 mg/kg rapamycin or vehicle starting on day 6 post-morphine injection. The tail flick test was performed before morphine injection and at 0.5 h after s.c. injection of an inducing dose of morphine (10 mg/kg) in naive mice on mornings 1, 3, 5, 8 and 10 in naive mice. The cold and mechanical tests were performed before morphine injection, on day 6 before rapamycin/vehicle injection and on day 11 after first morphine injection.

Western Blot Assay

The lumbar enlargement segments of spinal cord were collected. The tissues were homogenized with ice-cold lysis buffer (10 mM Tris, 1 mM phenylmethylsulfonyl fluoride, 5 mM $MgCl_2$, 5 mM EGTA, 1 mM EDTA, 1 mM DTT, 40 μM leupeptin, and 250 mM sucrose) with protease and phosphatase inhibitor cocktail (Sigma-Aldrich). After the crude homogenate was centrifuged at 4 °C for 15 min at 1000×*g*, the supernatants were collected for cytosolic proteins. After measuring protein concentration, the samples were heated for 5 min at 99 °C and loaded onto a 7.5% separating SDS–polyacrylamide gel. The proteins were then electrophoretically transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). After being blocked with 3% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h, the membranes were then incubated with following primary antibodies overnight. These antibodies included rabbit anti-p-mTOR (Ser2448, 1:1000, Cell Signaling Technology, Danvers, MA), rabbit anti-mTOR (1:1000, Cell Signaling Technology), rabbit anti-p-4E-BP1 (1:1000, Cell Signaling Technology), rabbit anti-4E-BP1 (1:1000, Cell Signaling Technology), and mouse anti-*β*-actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were detected by horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:3000, Jackson ImmunoResearch) and visualized by western peroxide reagent and luminol/enhancer reagent (Clarity Western ECL Substrate, Bio-Rad) and exposure using the ChemiDoc XRS System with Image Lab software(Bio-Rad). The intensity of blots was quantified with densitometry using Image Lab software (Bio-Rad). The average blot density from the control groups was set as 100%. The relative density values from time points or the treated groups were determined by dividing the optical density values from these groups by the average value of the control groups after each was normalized to the corresponding *β*-actin.

Statistical Analysis

All results are given as mean ± SEM. The number of mice in each group is 10. Sample size was based on the preliminary experiments and calculated using Sigmaplot 12.5 software. Data were analyzed using one-way or repeated measure two-way ANOVA followed by Tukey post hoc tests. Statistical tests were conducted using Graph Pad Prism 5.0 software. *P* values of less than 0.05 were considered statistically significant.

Results

Systemic Administration of Rapamycin Attenuates the Development and Maintenance of Morphine Analgesic Tolerance

We first examined the effect of systemic rapamycin on the development of morphine tolerance in mice. Morphine tolerance was induced by receiving subcutaneous injection of 20 mg/kg morphine twice a day for 5 days. Co-intraperitoneal injection of rapamycin (2.5 mg/kg) or vehicle (50% DMSO) once daily starting at day 0 before morphine for 5 days. In morphine plus vehicle group, morphine analgesic effects, as shown by MPAEs, were decreased by 29.7% and 92.8%, respectively, on days 3 and 5 after first injection of morphine, compared to the corresponding baseline (day 1) (Fig. 1a). In contrast, morphine's MPAEs in the morphine plus rapamycin group were reduced by 13.3% and 52.7%, respectively, on days 3 and 5 after first injection of morphine, compared to the corresponding baseline (Fig. 1a). The difference in morphine's MPAEs between these two

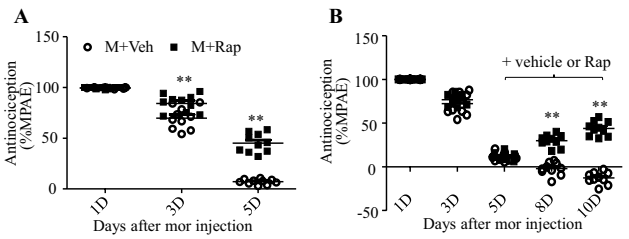

Fig. 1 Systemic administration of rapamycin attenuated the development (**a**) and maintenance (**b**) of chronic morphine tolerance. **a** Mice received subcutaneous injection of 20 mg/kg morphine (M) twice a day and intraperitoneal injection of 2.5 mg/kg rapamycin (Rap) or vehicle (Veh: 50% DMSO) once a day for 5 days. Tail-flick test was carried out before morphine injection, and 30 min after morphine injection in the morning of day 1, 3 and 5. N = 10/group. Two way RM ANOVA followed by Tukey post hoc test. $F_{(2,59)} = 50.57$, $^{**}P < 0.01$ versus the morphine and vehicle group at the corresponding time point. **b** Mice received subcutaneous injection of 20 mg/kg twice a day for 10 days and also intraperitoneal injection of 2.5 mg/kg rapamycin or vehicle starting on day 6 post-morphine injection. Tail-flick test was carried out before morphine injection, and 30 min after morphine injection in the morning of day 1, 3, 5, 8, and 10. N = 10/group. Two way RM ANOVA followed by Tukey post hoc test. $F_{(4,99)} = 116.97$, $^{**}P < 0.01$ versus the morphine and vehicle group at the corresponding time point

groups was significant at 5 days after first morphine injection ($P < 0.01$, Fig. 1a).

To examine the role of systemic rapamycin in the maintenance of morphine analgesic tolerance, we intraperitoneally injected rapamycin once daily starting at day 6 after first morphine injection for 5 days in mice that received s.c. injection of morphine twice a day for 10 days. Morphine lost analgesic effect completely on days 8 and 10 post-first morphine injection in the morphine plus vehicle group (Fig. 1b), whereas MPAEs were 26.9% and 37.8%, respectively, on days 8 and 10 post-first morphine injection in the morphine plus rapamycin group (Fig. 1b). There were marked differences in the morphine's MPAEs at these two time points between two groups ($P < 0.01$, Fig. 1b).

Systemic Administration of Rapamycin Blocks Morphine-Induced Cold Allodynia During the Development and Maintenance Periods

We next examined the effect of co-intraperitoneal injection of rapamycin (once a day for 5 days) on the development of cold allodynia (as shown by the decrease in paw withdrawal latency (PWL) in response to cold stimulation) caused by subcutaneous morphine injection twice a day for 5 days. The PWL decreased significantly at day 6 (8.17 ± 0.44 s) compared to day 1 (13.61 ± 0.52 s) in the morphine plus vehicle group ($P < 0.01$, Fig. 2a). The PWL also decreased from 13.14 ± 0.44 s on day 1 to 10.72 ± 0.32 s on day 6 in the morphine plus rapamycin group ($P < 0.01$, Fig. 2a). It is evident that the PWL increased significantly in the morphine plus rapamycin group compared to the morphine plus vehicle group on day 6 after first morphine injection ($P < 0.01$, Fig. 2a). As shown in Fig. 2b, systemic rapamycin also partially relieved morphine-induced cold allodynia when rapamycin was given starting on day 6 post-morphine injection. The PWL increased significantly in the morphine plus rapamycin group (9.82 ± 0.49 s) compared to the morphine plus vehicle group (7.54 ± 0.57 s) on day 11 ($P < 0.01$, Fig. 2b) after first morphine injection. These data suggest that systemic rapamycin relieved morphine-induced cold allodynia in both development and maintenance periods.

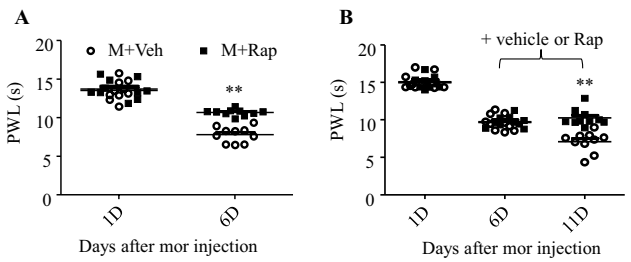

Fig. 2 Systemic administration of rapamycin attenuated the development (**a**) and maintenance (**b**) of morphine-induced cold allodynia. **a** Mice received subcutaneous injection of 20 mg/kg morphine (M) twice a day and intraperitoneal injection of 2.5 mg/kg rapamycin (Rap) or vehicle (Veh: 50% DMSO) once a day for 5 days. Cold plate test was carried out prior to morphine injection and day 6 after morphine injection. N = 10/group. Two way RM ANOVA followed by Tukey post hoc test. $F_{(1,39)} = 37.29$, $^{**}P < 0.01$ versus the morphine + vehicle group at the corresponding time point. **b** Mice received subcutaneous injection of 20 mg/kg twice a day for 10 days and also intraperitoneal injection of 2.5 mg/kg rapamycin or vehicle starting on day 6 post-morphine injection. Cold plate test was carried out prior to morphine injection, in the morning of day 6 before morphine injection, and day 11 after morphine injection. N = 10/group. Two way RM ANOVA followed by Tukey post hoc test. $F_{(2,59)} = 15.03$, $^{**}P < 0.01$ versus the morphine + vehicle group at the corresponding time point

Systemic Administration of Rapamycin Blocks Morphine-Induced Mechanical Allodynia During the Development and Maintenance Periods

Repeated s.c. morphine applications (twice a day for 5 days) also led to the development of mechanical allodynia demonstrated by significant reductions in paw withdrawal frequency (PWF) in response to 0.07 g von Frey filament stimulation in the morphine plus vehicle group on days 6 and 11 after first morphine injection on both sides ($^{*}P < 0.05$ vs. the corresponding baseline; Fig. 3a–d). These reductions were significantly attenuated by co-administration of rapamycin in the morphine plus rapamycin group ($^{*}P < 0.05$ vs. the corresponding time points in the morphine plus vehicle group; Fig. 3a–d). These data indicate that systemic administration of rapamycin blocks morphine-induced mechanical allodynia during the development (Fig. 3a, b) and maintenance periods (Fig. 3c, d).

Systemic Administration of Rapamycin Blocks Morphine-Induced Spinal mTORC1 Activation

Consistent with continuous intrathecal morphine injections [6], subcutaneous injection of 20 mg/kg morphine also induced spinal mTORC1 activation as demonstrated by significant increases in the expression of p-mTOR and p-4E-BP1 (one of mTOR's downstream targets) in spinal cord dorsal horn (Fig. 4a, b). Compared to the saline plus vehicle group, the levels of p-mTOR and p-4E-BP1 were increased 1.96- and 1.86 -folds, respectively, in the morphine plus vehicle group ($^{*}P < 0.05$; Fig. 4a, b). Intraperitoneal administration of rapamycin in the morphine plus rapamycin group completely reversed the increases in p-mTOR and p-4E-BP1 expression in dorsal horn, compared to the morphine plus vehicle group ($^{\#}P < 0.05$, Fig. 4a, b). In contrast, the expression of p-mTOR and p-4E-BP1 in the morphine plus ascomycin (an analogue of rapamycin, but no effect on mTOR activity [6, 14]) group still remained a remarkable increase. No statistical difference was found between the morphine plus vehicle group and the morphine

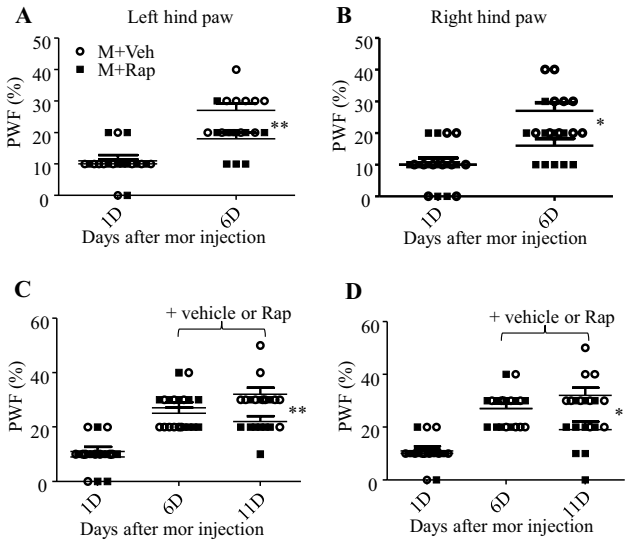

Fig. 3 Systemic administration of rapamycin attenuated the development (**a, b**) and maintenance (**c, d**) of morphine–induced mechanical allodynia. **a, b** Mice received subcutaneous injection of 20 mg/kg morphine (M) twice a day and intraperitoneal injection of 2.5 mg/kg rapamycin (Rap) or vehicle (Veh: 50% DMSO) once a day for 5 days. Mechanical test was carried out prior to morphine injection and day 6 after morphine injection. N = 10/group. Two way RM ANOVA followed by Tukey post hoc test. (**a**, left hind paw), $F_{(1,39)} = 10.98$, $^{**}P < 0.01$ versus the morphine + vehicle group at the corresponding time point. (**b**, right hind paw), $F_{(1,39)} = 4.45$, $^{*}P < 0.05$ versus the morphine + vehicle group at the corresponding time point. **c, d** Mice received subcutaneous injection of 20 mg/kg twice a day for 10 days and also intraperitoneal injection of 2.5 mg/kg rapamycin or vehicle starting on day 6 post-morphine injection. Mechanical test was carried out prior to morphine injection, in the morning of day 6 before morphine injection, and day 11 after morphine injection. N = 10/group. Two way RM ANOVA followed by Tukey post hoc test. (**a** left hind paw), $F_{(2,59)} = 5.67$, $^{**}P < 0.01$ versus the morphine + vehicle group at the corresponding time point. (**b** right hind paw), $F_{(2,59)} = 4.01$, $^{*}P < 0.05$ versus the morphine + vehicle group at the corresponding time point

plus ascomycin group (Fig. 4). The administration of rapamycin alone in the saline plus rapamycin group did not affect the basal expression of p-mTOR and p-4E-BP1 (Fig. 4). These results suggest that spinal mTORC1 is one of targets for systemic rapamycin application.

Discussion

In the present study, we demonstrated that systemic rapamycin application delayed morphine analgesic tolerance and attenuated morphine-induced cold and mechanical allodynia through inhibiting spinal mTORC1 activation in mice. Combined with previous findings which showed the consistent beneficial effect of intrathecal infusion of rapamycin on the development and maintenance of chronic morphine tolerance and hyperalgesia in naive and neuropathic pain rats [6, 9], spinal mTORC1 may play a critical role in opioid tolerance and hyperalgesia.

The mechanisms of morphine tolerance and hyperalgesia are usually considered to come from adaptive changes within the peripheral and central nervous systems [10]. These changes involved adaptive modifications of the μ-opioid receptor, particularly desensitization and downregulation, as well as activation of the anti-opioid system [3, 10, 15], finally converging on the protein translation process [10, 14]. Repeated intrathecal injections of morphine increased translational activity and new protein synthesis in the spinal dorsal horn, which could be blocked by spinal cord mTORC1 inhibition via intrathecal infusion of rapamycin [6, 9]. Here we showed that systemic rapamycin had the equivalent effect on morphine-induced analgesic tolerance and hyperalgesia in mice. Previous study has shown that chronic morphine-induced dorsal horn mTOR activation occurs through the μ-opioid receptor-triggered PI3K/AKT pathway. The activated mTORC1 phosphorylates its downstream targets such as 4E-BPs and p70 ribosomal S6 protein kinases (S6Ks), and gates the synthesis of many individual proteins that participate in the development and maintenance of morphine tolerance and hyperalgesia [6].

mTORC1 in nervous system plays a key role in morphine tolerance and hyperalgesia. Like intrathecal administration of morphine in the previous studies [6, 9], subcutaneous injection of morphine in the present study also activated mTORC1 and its downstream pathway in spinal cord. Systemic administration of rapamycin not only blocked this activation but also attenuated the morphine tolerance and hyperalgesia. These findings suggest that spinal mTORC1 may participate in the mechanisms underlying the morphine tolerance and hyperalgesia. It should not be excluded that systemic rapamycin may also target to other pain related area, such as dorsal root ganglion and brain regions. mTOR and S6K1 are highly expressed not only in spinal cord dorsal horn neurons, but also in small DRG neurons [16]. Moreover, chronic inflammation caused by complete Freund's adjuvant (CFA)-induced upregulation of p-mTOR in DRG. Rapamycin can relieve inflammatory pain by inhibiting mTORC1 activation in DRG and spinal cord [17]. These data suggest that DRG may also be a target of systemic rapamycin. In addition, mTOR signaling has been found play a role in morphine-induced adaptations in ventral tegmental area dopamine neurons [18]. Another study showed that mTOR signaling in the hippocampus is essential for the acquisition of morphine-induced place preference induction [19]. These findings indicate the broad function of mTOR signaling. The analgesic effect of systemic rapamycin by targeting mTORC1 signaling in other brain regions cannot be excluded.

In conclusion, our findings demonstrated that systemic administration of rapamycin had the effect on morphine

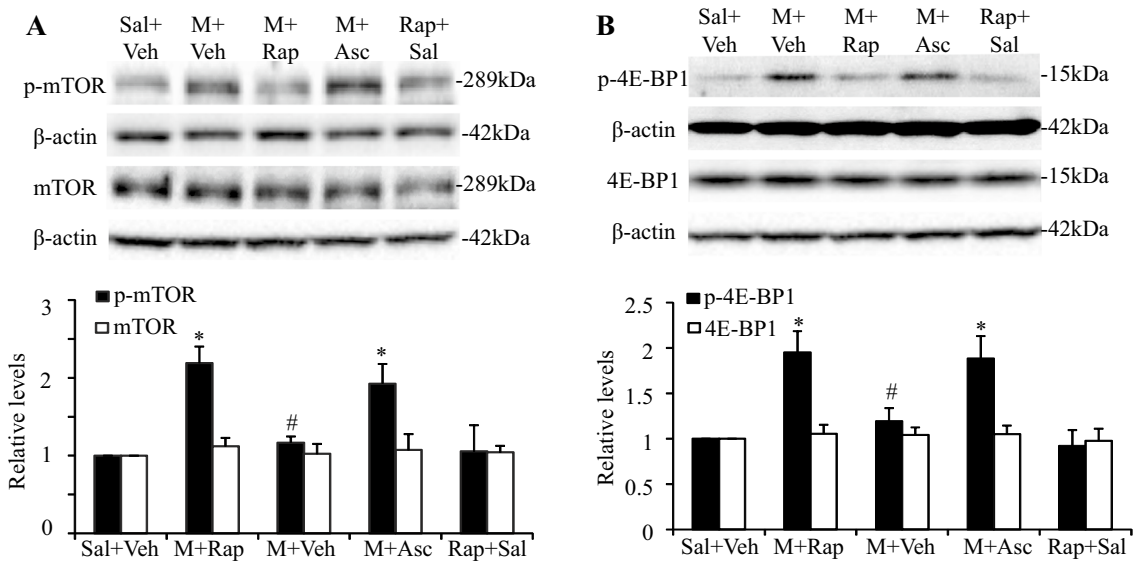

Fig. 4 Systemic rapamycin reversed the upregulation of p-mTOR and p-4E-BP1 expression in spinal dorsal horn. Mice received subcutaneous injection of 20 mg/kg morphine (M) twice a day and intraperitoneal injection of 2.5 mg/kg rapamycin (Rap) or vehicle (50% DMSO) once a day for 5 days. All lumbar spinal dorsal horns were harvested and p-mTOR and mTOR (**a**), p-4E-BP1 and 4E-BP1 (**b**) were examined by western blots. N = 3/group, $^{*}P < 0.05$ versus the Sal+Veh group; $^{\#}P < 0.05$ versus the M+Veh group. Sal: saline; Veh: vehicle; M: morphine; Rap: rapamycin; Asc: ascomycin

tolerance and hyperalgesia at least in part through inhibition of spinal mTORC1 and its downstream signaling activation. As the mTORC1 inhibitors (e.g. rapamycin analog) are FDA-approved clinical drugs and orally are administered for organ transplantation and cancer treatment, our findings strongly suggest that oral administration of the mTORC1 inhibitors may be promising drugs for use as adjuvants with opioids in treating chronic pain in the future. However, in clinic trials as of its immunosuppressive function to prevent of organ transplant rejection and for the treatment of lymphangioleiomyomatosis, some side effects have been observed which include peripheral edema, hypercholesterolemia, abdominal pain, headache, nausea, diarrhea, etc [20]. These symptoms should be carefully monitored when rapamycin were used to prevent morphine-induced analgesic tolerance and hyperalgesia in the future.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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