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Original Article

# Melanocortin 4 Receptor Is Involved in the Development of Morphine Tolerance

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## ABSTRACT

**Background:** Prolonged morphine treatment usually results in the development of analgesic tolerance. Melanocortin 4 receptor (MC4R) is involved in the development of morphine tolerance. The aim of this study was to examine the effects of an MC4R antagonist, HS014, on MC4R-mediated hyperalgesia and on microglia and cytokine expression in the spinal cord of rat during morphine tolerance.

**Methods:** Thirty rats were assigned randomly to the N group, M group, HM group, NM group and HN group (N=6 in each group). Rats received 5-day treatment with saline (N group) or morphine (M group). Rats were given an intrathecal injection (i.t.) of HS014 (HM group) or saline (NM group) at 15 minutes prior to the morphine challenge. In the HN group, rats were injected with HS014 at 15 minutes prior to the saline. To observe the effect of HS014 on the development of morphine tolerance, morphine-tolerant rats were assigned to M1 group, M2 group, M3 group and N group on day 6 (N=6 in each group). The morphine-tolerant rats in M1 group, M2 group and M3 group received morphine (10 mg/kg, i.p.), HS014 (5 $\mu$g, i.t.) and HS014 (5 $\mu$g, i.t.) followed 15 minutes later by injection of morphine (10 mg/kg, i.p.), respectively. Control rats (N group, N=6) were injected with saline under identical conditions. Hot-plate test and immunochemistry were used to examine the withdrawal latency and inflammatory cytokines.

**Results:** Morphine treatment (10 mg/kg, i.p. twice daily) over 5 days induced tolerance as reflected by a significant reduction of withdrawal latency from 29.67 ± 1.81 s to 8.67 ± 1.70 seconds in the hot-plate test. Repeated HS014 injection prior to morphine administration inhibited the development of morphine tolerance. Furthermore, a single administration of HS014 restored morphine analgesic potency in morphine-tolerant rats. Immunohistochemical staining showed that the administration of HS014 during the induction of morphine tolerance inhibited the activation of microglia, reduced the expression of proinflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α, and increased the expression of anti-inflammatory cytokines (IL-10) at the L5 lumbar spinal cord.

**Conclusions:** HS014 attenuates the development of antinociceptive tolerance following chronic administration of morphine by inhibiting microglial activation and reducing the expression of IL-1, IL-6 and TNF-α.



Evidence Based Communications

While morphine is used for various types of pain as a highly potent analgesic effect, its efficacy gradually subsides after prolonged administration, leading to the development of morphine tolerance (1). The underlying mechanisms for morphine tolerance remain largely unknown so far despite of extensive research over the years. Previous studies have shown that systemic morphine administration activates glial cells, including microglia and astrocytes (2-5). Microglia activation, enhanced expression of proinflammatory cytokines and decreased expression of anti-inflammatory cytokines are present in the lumbar spinal cord of rats after prolonged morphine treatment (4, 6). Inhibition of glial activation and proinflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α can attenuate the development of morphine tolerance in rats (4, 7).

Melanocortins, a group of peptide hormones derived from proopiomelanocortin in the pituitary gland, exert various physiological functions such as pigmentation, energy homeostasis and inflammation via binding to the melanocortin receptors (8). In the central nervous system, melanocortins are involved in the induction of neural plasticity (9). Melanocortin 4 receptor (MC4R) has been reported to play an important role in neuropathic pain and the development of morphine tolerance (10, 11). Vrinten et al. (12) have demonstrated the antiallodynic action of an MC4R antagonist SHU9119 when administered into the cisterna magna. Moreover, SHU9119 has also been shown to enhance morphine analgesic effects in neuropathic animal models (11). Previous studies suggested that neuropathic pain and the development of morphine tolerance shared similar mechanisms (13), thus prompting the idea that MC4R may be a potential target for the inhibition of morphine tolerance.

In the present study, we investigated the effect of MC4R antagonists on antinociceptive response, microglia activation and cytokines expression in the development of chronic morphine tolerance.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley (SD) rats bred at Qingdao Drug Research Institute, China, weighing 220-250 g, were used for this study. The rats were housed in single cages under standard conditions (12-h light/dark cycle, 24 ± 2 ℃; 50-70% humidity) with free access to food and water. All experimental procedures in this study were approved by the Bioethics Committee of the Institute of Pharmacology and were performed in accordance with the ethical guidelines for investigations of experimental pain in conscious animals (14).

### Intrathecal Catheter Implantation and Drug Administration

Intrathecal (i.t.) catheters were implanted as described previously by Yaksh and Rudy (15). Briefly, the rats were anesthetized with 10% chloral hydrate (400 mg/kg, i.p.). After exposing to the cisternal membrane, i.t. polyethylene catheters (PE-10: OD 0.5 mm, ID 0.25 mm, Anilab, Ningbo, China) were inserted via an incision in the cisterna magna and advanced caudally to the lumbar enlargement of the spinal cord (approximately 7-8 cm deep). The catheter was fixed firmly under the skin and was sealed effectively. After the surgery, the rats were housed individually, and were allowed to recover for at least 7 days before habituation and behavioral testing. Animals with neurological deficits (signs of paralysis) or infection were excluded from experiments. The proper location of the catheter was confirmed by assessing sensory and motor blockade after intrathecal injection of 10 μl of 2% lidocaine. At the end of the study, location of the catheter was examined by postmortem dissection. An initial injection of saline (10 μl) was delivered through the catheter, and the catheter was then tightened. Subsequently, for individual experiments, drugs (in 5 μl solution) as specified individually were slowly injected through the catheter within 1-2 minutes, with 5 μl saline injected into the control rats, followed by 10 μl saline to flush the catheter.

To establish the role of HS014 in the development of morphine tolerance, thirty rats were assigned randomly to the N group, M group, HM group, NM group and HN group (N=6 in each group). Rats received 5-day treatment with saline (N group) or morphine (M group). Rats were given intrathecal injection of 5 μg of HS014 (HM group) or saline (NM group) at 15 minutes prior to the morning morphine challenge. In the HN group, rats were injected with 5 μg of HS014 at 15 minutes prior to the saline. Morphine was administered intraperitoneally (10 mg/kg) twice daily at 8 AM and 5 PM for five consecutive days.

In order to determine the influence of HS014 injection on morphine tolerance, morphine-tolerant rats were established by consecutive injection of morphine for 5 days. Morphine-tolerant rats were assigned to M1 group, M2 group, M3 group and N group on day 6 (N=6 in each group). The morphine-tolerant rats in M1 group, M2 group and M3 group received morphine (10 mg/kg, i.p.), HS014 (5 μg, i.t.) and HS014 (5 μg, i.t.) followed 15 minutes later by injection of morphine (10 mg/kg, i.p.), respectively. Control rats (N group, N=6) were injected with saline under identical conditions.

### Thermal Test

Antinociceptive response to morphine was determined by the hot-plate test. Animals were placed on a hot metallic plate surface maintained at 55 ℃. The time until the occurrence of either licking of the hind paws, shaking, or jumping off the surface was recorded as reaction time, with the cutoff time setting at 60 seconds. Withdrawal latencies to thermal stimulation were determined at 30 minutes after the second injection of morphine.

### Immunochemistry

Five days after the thermal test, the rats were euthanatized by deep anesthesia of 10% chloralhydrate. Rats were then perfused with phosphate-buffer saline. Laminectomy was performed from the lower edge of the twelfth thoracic vertebrae to the sacral vertebrae. The L5 lumbar spinal cord was collected, frozen immediately in liquid nitrogen and stored at -80 ℃. The tissues were fixed in 4% paraformaldehyde at 4 ℃ overnight until the tissues sink to the bottom. The samples were fixed in 10% paraformaldehyde for 12 hours and then were washed three times with 0.1 mol/L phosphate-buffer saline (PBS) for 120 minutes. Then the sample was dehydrated from 70% to 80%, 90%, 95% and 100% ethanol, and then was hyalinized with dimethylbenzene and imbedded with paraffin wax.

The spinal cord was cut into 5 μm-thick sections using a Microm cryostat (Microm International GmbH, Walldorf, Germany). The tissue sections were stained for assessment of OX-42, TNF-α, IL-1, IL-6, and IL-10 expression according to respective immunohistochemistry kit supplied by Wuhan Boster Biological Technology (Wuhan, China). The details are as described previously by Chu (10). Three sections per rat and three squares located at the same area of the dorsal horn in each section were counted. The total number of positive cells in each dorsal horn section was calculated under the high microscope (400 × magnification) for evaluation of OX-42, TNF-α, IL-1, IL-6, and IL-10-immune response (IR) by an experimenter who did not know which treatment the rat was receiving.

### Statistical Analysis

All statistical analyses were performed using the SPSS 13.0 software. Values from the thermal test were represented as means ± SEM, and values from cell counts as means ± SD. A sample size of 6 rats per group was selected according to our preliminary results. All quantitative data were examined for their distribution before comparision, such as normally distributed variables (withdrawal latency), the number of OX- 42, cytokines number. Behavior tests were analyzed by repeated measures one way ANOVA. Post- drug time course measures for thermal hyperalgesia were analyzed by two-way repeated measures ANOVA (post hoc, Newman–Keuls), while the TNF-α, IL-1β, IL- 6, and IL- 10 contents were analyzed by one- way ANOVA (Dunnett multiple comparisons). Differences among means were considered to be statistically significant if $P<0.05$.

## RESULTS

### Development of Morphine Tolerance

The baseline withdrawal latency (before morphine administration) remained constant throughout the entire testing procedure in the N group (Figure 1). On day 1 and 2, morphine administration induced significant analgesia in the M group compared to the N group ($P<0.001$). On day 4 of morphine administration, a significant decrease in the analgesic efficacy was observed, indicating that 5- day intrathecal administration of morphine induced morphine tolerance. On the other

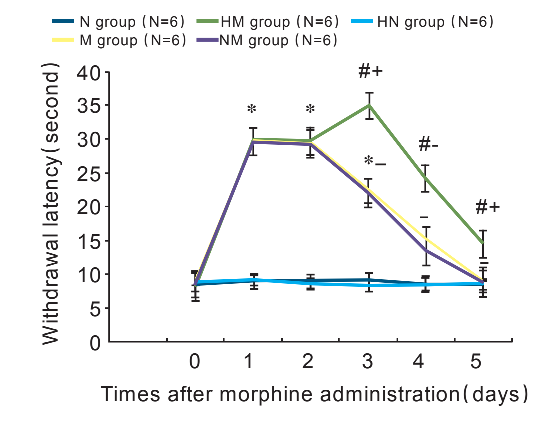


**Figure 1. Effect of HS014 Pretreatment on the Analgesic Effect of Morphine.**
Data are expressed as means ± SEM. *P < 0.001 compared to normal saline group (N); -P < 0.05 compared to morphine group (M) on day 1; #P < 0.05 compared to morphine group (M); + P < 0.05 compared to HS014 + morphine group (HM) on day 1.

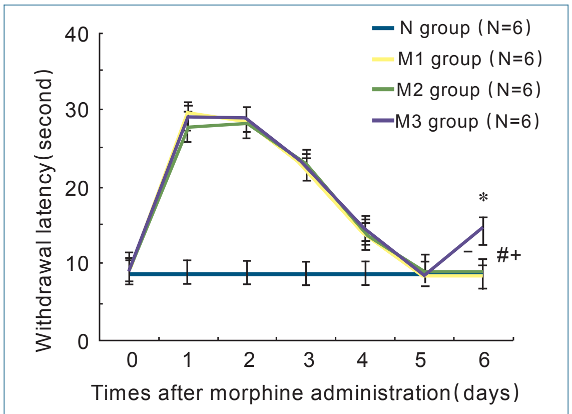


**Figure 2. Effect of HS014 Single Injection on Morphine Tolerance.**
Data are expressed as means±SEM. *P<0.05 compared to morphine group (M1); +P>0.05 compared to M1; #P > 0.05 and -P > 0.05 compared to normal saline group (N).

hand, the withdrawal latency of the M group did not differ significantly from that of the N group. Saline delivered via the intrathecal catheter prior to morphine (i.p.) did not significantly alter the effect of morphine (P > 0.05).

### Effect of HS014 Administration on Morphine-Induced Antinociception

Repeated administration of HS014 (5 μg, i.t.) did not alter the withdrawal latency in rats in the N group (Figure 1). Two-way ANOVA revealed significant effect of intrathecal injection of HS014 (withdrawal latency: F1, 29= 40.081, P<0.001) and interaction between HS014 treatment and time (withdrawal latency: F5, 29= 6.383, P<0.001). HS014-induced changes in development of morphine tolerance are shown in figure 1. Morphine-induced analgesia was similar between the M group and the HM group on day 1 and 2 (P>0.05). However, the withdrawal latency reduced significantly in the M group after day 3, while the withdrawal latency was markedly extended after day 3 in the HM group compared to the initial effect of morphine administration alone (P<0.05).

### Effect of Single I.T. HS014 Injection on Withdrawal Latency in Morphine-Tolerant Rats

After five days of morphine administration, the rats were tolerant to morphine as shown above. On day 6, morphine (10 mg/kg, i.p.), HS014 (5 μg, i.t.), or HS014 (5 μg, i.t.) was administered to the morphine-tolerant rats at 15 minutes prior to morphine, with saline injection as the control. HS014 (5 μg, i.t.) injected at 15 minutes prior to morphine resulted in a significant increase in the withdrawal latency (P<0.05) compared to morphine alone (Figure 2), while injection of HS014 alone showed no antinociceptive effect (P>0.05) on morphine-tolerant rats (Figure 2).

### Effect of HS014 on Microglia (OX-42) Immunoreactivities in the Dorsal Horn of the Lumbar Spinal Cord of Morphine-Tolerant Rat

The expression of OX-42 at L5 lumbar spinal cord in M group was significantly higher compared to the N group (Figure 3). HS014 had no effect on the expression of OX-42 in the N group. In contrast, HS014 injection during the induction of morphine tolerance significantly attenuated the morphine-induced upregulation of OX-42 (Figure 3).

### Effect of HS014 on IL-1, IL-6, IL-10, and TNF-α Expression in Rat Spinal Cords

In the M group, chronic administration of morphine significantly increased the expression of IL-1, IL-6, and TNF-α but decreased the expression of IL-10 in L5 lumbar spinal cord compared to the N group (Table). HS014 had no effect on the expression of IL-1, IL-6, TNF-α or

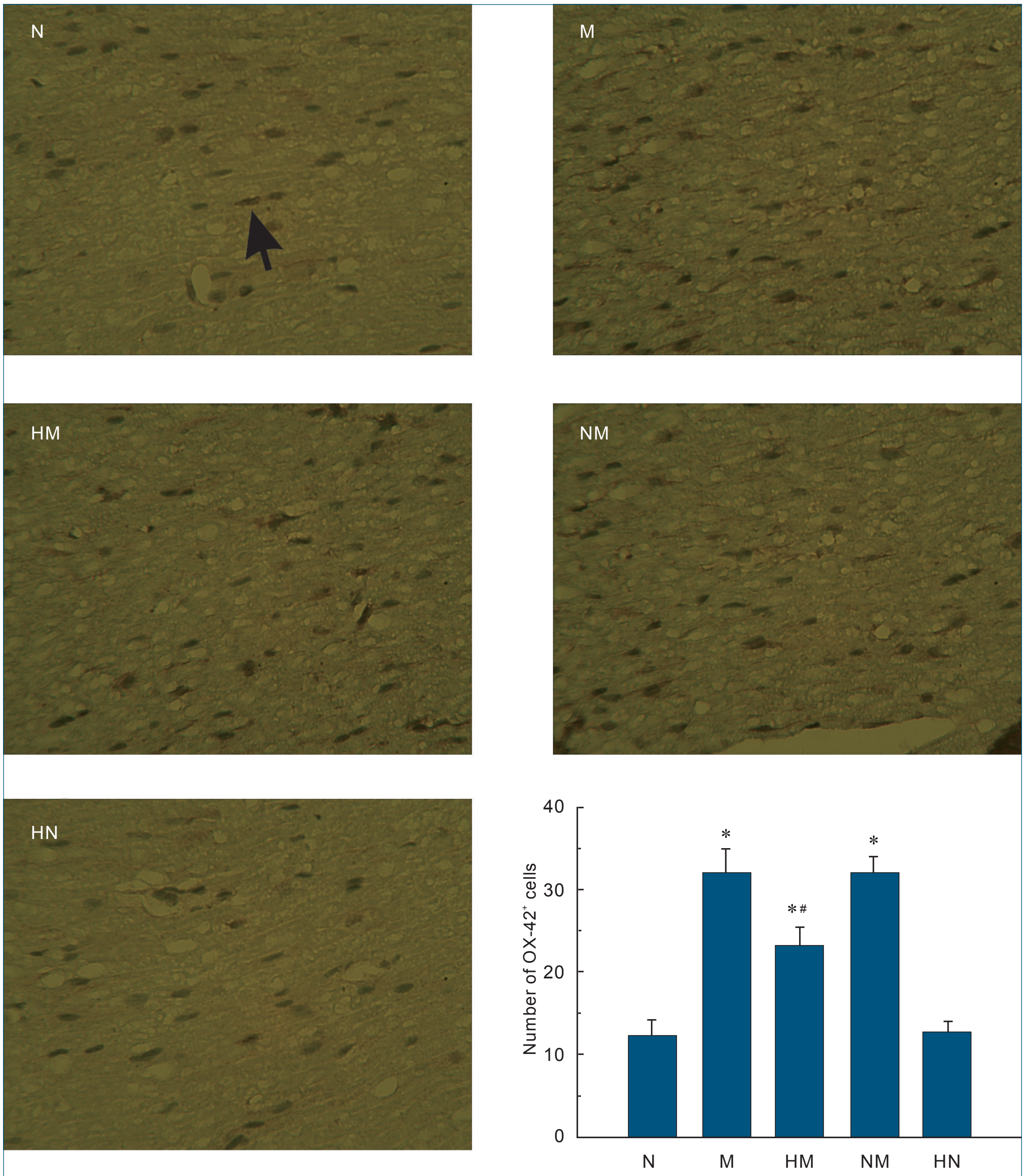


**Figure 3. Effect of HS014 on Glial Activation in the Spinal Cord of Morphine-Tolerant Rats Determined by OX-42 Expression.**
The arrow points to an OX-42 positive cell. Data are expressed as means ± SD. *P<0.05 compared with N group; #P < 0.05 compared with M group.

IL-10 in the N group. However, HS014 treatment during the induction of morphine tolerance significantly attenuated the morphine-induced upregulation of IL-1, IL-6, and TNF-α and downregulation of IL-10 expression (Table).

## DISCUSSION

The present study investigated the contribution of HS014(MC4R antagonist) to the development

Table. Comparison of Cytokine Expression in the Spinal Cord (number, means±SD).

| Group | TNF positive cell | IL-1 positive cell | IL-6 positive cell | IL-10 positive cell |
|---|---|---|---|---|
| N(N=6) | 28.4±1.1 | 48.5±0.5 | 38.0±0.5 | 69.9±1.2 |
| M(N=6) | 99.3±2.1* | 97.8±1.3* | 68.1±0.5* | 71.2±1.3* |
| HM(N=6) | 61.0±1.3☆ | 64.3±1.2☆ | 49.1±0.7☆ | 126.8±2.2☆ |
| NM(N=6) | 92.6±1.4#◇ | 96.9±0.7#◇ | 69.1±0.7#◇ | 70.4±0.9#◇ |
| HN(N=6) | 26.1±0.8** | 49.4±0.5** | 38.4±0.7** | 70.0±1.0** |

*P<0.001, #P<0.001, **P>0.05 compared to normal saline group (N); ◇ P>0.05, ☆ P<0.001 compared to morphine group (M).

of morphine-induced tolerance and its biochemical mechanisms. Results showed that administration of HS014 decreased the development of morphine tolerance and its associated hyperalgesia. This findings were attributed to the modulation of blockade of MC4 receptors on morphine-induced microglia activation and inflammatory cytokines secretion. The findings of this study has shed light on the mechanisms underlying the development of morphine tolerance, and provided a potential target (MC4R) as a new strategy for the treatment of morphine tolerance.

Since morphine is still the most effective drug for the treatment of chronic pain, it is very important to maintain and/or resume the potency of opioid analgesia. Reduction or loss of analgesic potency has been a major limitation in the repeated use of morphine, making chronic pain obstinate in clinical practice.

Currently, the interest is centered on the investigation of morphine tolerance. However, the mechanisms of the development of morphine tolerance are not completely identical. For example, 5-hydroxytryptamine exerts opposite action on the development (16) and maintenance (17) of opioid tolerance while the activation of GABA (18) and dopamine (19) receptors is only involved in the development of tolerance. Therefore, it is necessary to investigate the mechanisms underlying morphine tolerance. Accumulating evidence suggests that MC4R plays an important role in morphine tolerance and neuropathic pain (12, 16). Recently, expression of MC4R is also found in dorsal root ganglion (DRG) neurons (17, 18) and microglia (3), which is involved in the development and maintenance of neuropathic pain. Similar mechanisms are implicated in the development of morphine tolerance and neuropathic pain. Previous studies by our group have shown that MC4R induced hyperalgesia and allodynia by activation of p38 MAPK in DRG neurons (16). The present study found that morphine tolerance was markedly delayed by pretreatment with HS014, which provided further evidence that MC4R contributed to the development of morphine tolerance. This study also found that HS014 could attenuate the decreased analgesic effect of morphine caused by tolerance. Based on these findings, we hypothesize that MC4R enhances activity of the opioid systems.

Morphine has been demonstrated to activate spinal glial activity, enhance production of TNF-α and nitric oxide, and inhibit microglial chemotaxis (5, 19). Morphine tolerance is associated with spinal microglial and astroglial activation (3, 11, 20). Inhibition of spinal glial activation can reverse the development of morphine tolerance in rats (5). Consistently, the present study has shown that morphine tolerance could induce microglial activation in the spinal cord that is associated with increased expression of OX-42-IR, and HS014 could suppress morphine-induced spinal microglial activation, suggesting that MC4R may be involved in the establishment of spinal microglial plasticity.

Inflammatory cytokines and MC4R both play an important role in the development and maintenance of morphine tolerance (21). The chronic administration of morphine leads to upregulation of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α (4, 22). In our previous study (10), we have demonstrated that activation of MC4R in periaqueductal gray after peripheral nerve injury participated in pain facilitation by regulating the glial activation and inflammatory cytokines secretion. In the present study, we found that HS014 inhibited the upregulation of these proinflammatory cytokines (IL- 1, IL- 6, and TNF-α) in the spinal cord induced by repeated morphine administration. These results further support the theory mentioned above and indicated that intrathecal delivery of HS014 could attenuate the development of spinal antinociceptive tolerance to morphine via anti-inflammatory and pro-resolving actions.

## CONCLUSIONS

HS014 has shown a profound attenuating effect on the development of antinociceptive tolerance following chronic administration of morphine. This effect may be mediated by inhibiting microglial activation, and reducing the expression of IL-1, IL-6, and TNF-α. Therefore, it can be proposed that MC4R could be an effective therapeutic target to restore the potency of morphine following the development of tolerance.

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The authors report no other conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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