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# Repeated Morphine Administration Increases *TRPV1* mRNA Expression and Autoradiographic Binding at Supraspinal Sites in the Pain Pathway

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## Abstract

Repeated morphine administration induces tolerance to its analgesic effects. A previous study reported that repeated morphine treatment activates transient receptor potential vanilloid type 1 (TRPV1) expression in the sciatic nerve, dorsal root ganglion, and spinal cord, contributing to morphine tolerance. In the present study, we analyzed TRPV1 expression and binding sites in supraspinal pain pathways in morphine-tolerant mice. The *TRPV1* mRNA levels and binding sites were remarkably increased in the cortex and thalamus of these animals. Our data provide additional insights into the effects of morphine on TRPV1 in the brain and suggest that changes in the expression of, and binding to TRPV1 in the brain are involved in morphine tolerance.

**Key Words:** TRPV1, Morphine, RT-PCR, RTX, Autoradiography, Tolerance

## INTRODUCTION

Morphine, an alkaloid found in poppy seeds, reduces the excitability of cells transmitting pain information mainly by acting on μ-opioid receptors (Yu, 1996). These receptors are widely distributed in the peripheral and central nervous systems (Yaksh, 1997). By blocking pain transmission at the peripheral, spinal, and supraspinal levels, morphine has a powerful analgesic effect (Yaksh, 1997). Moreover, the potent analgesic effects of morphine result from the interaction of the spinal and supraspinal effects (Yeung and Rudy, 1980). However, a tolerance to morphine develops rapidly after repeated administration, constituting one of the main challenges posed by the use of morphine for the management of chronic pain. Currently, the molecular and cellular mechanisms underlying morphine tolerance are not completely understood. However, an increased release and expression of pain-associated molecules such as chemokines, pro-inflammatory cytokines (Hutchinson *et al*., 2008), pronociceptive neurotransmitters (Ma *et al*., 2000; Gardell *et al*., 2002; King *et al*., 2005; Yue *et al*., 2008), and transient receptor potential vanilloid type 1 (TRPV1) (Chen *et al*., 2008) in the spinal cord and peripheral sites of the pain pathway are widely accepted as causes of morphine tolerance.

TRPV1 is a nonselective cation channel that is activated by diverse painful stimuli, including noxious-heat, acidic pH, endogenous lipids, capsaicin (Tominaga *et al*., 1998), lipidic molecules (Hernandez-Garcia and Rosenbaum, 2014), and venomous animal peptide toxins (Bohlen *et al*., 2010; Hakim *et al*., 2015; Yang *et al*., 2015). The TRPV1 channel has a homotetrameric structure, in which each subunit possesses, inclusive of a pore-forming loop between TM5 and TM6, several ankyrin repeat domains at the N terminus, six transmembrane domains (TM1–6), and a large intracellular C terminus containing a conserved amino acidic sequence called the “TRP box” (Liao *et al*., 2013). This channel is mainly expressed in the primary sensory neurons that play a crucial role in pain transmission (Caterina *et al*., 1997; Tominaga *et al*., 1998). The presence of TRPV1 in the brain is also documented (Szallasi and Di Marzo, 2000). Particularly, TRPV1 is found in many regions regulating pain transmission and modulation (Roberts *et al*., 2004; Toth *et al*., 2005; Starowicz *et al*., 2008). Moreover, it may participate in pain regulation in the central nervous system because TRPV1 antagonists with the ability to cross the blood–brain barrier were shown to be more potent (Cui *et al*., 2006). Despite intensive research regarding TRPV1, the

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role of brain TRPV1 in pain has not been well investigated.

Previous studies on mice and rats have reported a role of TRPV1 in morphine tolerance. Repeated morphine administration induces the upregulation of TRPV1 in the sciatic nerve, dorsal root ganglia (DRGs), and spinal cord, which contributes to morphine tolerance (Chen *et al*., 2008). However, whether repeated morphine treatment affects TRPV1 in supraspinal regions has not been investigated yet. Since the brain is involved in pain transmission and modulation, the present study aimed to assess changes in TRPV1 expression and binding in the mouse brain after repeated morphine treatment.

## MATERIALS AND METHODS

### Animals

Male ICR mice (22-25 g) were purchased from Dae Han Biolink Co., Ltd (Eumseong, Korea). Eight to ten mice were housed in each cage. Mice were acclimatized in an animal room under a 12/12 h light/dark cycle at 22°C ± 2°C for one week before the experiment onset. Mice were randomly distributed into experimental groups. All animal care procedures were conducted in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of the Sungkyunkwan University approved the study protocol (protocol approval number, SSKKUIACUC2018-04-19-1).

### Drugs

Morphine hydrochloride (Macfarlan Smith Ltd., Edinburgh, UK) was dissolved in physiological saline and 10 mL/kg were injected subcutaneously (s.c.) into mice at the indicated concentrations.

Tritiated [$^3$H]resiniferatoxin ([$^3$H]RTX, 37.2 Ci/mmol) was purchased from Perkin Elmer (Boston, MA, USA).

### Assessment of morphine tolerance

The treatment schedule was similar to our previously published schedule that induced morphine tolerance with slight modifications (Nguyen *et al*., 2010). At day 0, basal nociceptive response was determined for each mouse using a hot plate apparatus in a plastic cylinder (height, 20 cm, diameter, 14 cm). Mice were individually placed on the hot plate (52°C). The time taken for a mouse to lick its hind paw or jump was recorded (latency). A cut-off time of 40 s was set to prevent tissue damage. Thirty min after measuring the baseline latency, mice were injected acutely with saline or morphine (5 mg/kg, s.c.). Mice were tested again after 30, 60, 90, and 120 min. Then, mice were further injected with 10 mg/kg s.c. of morphine or saline once a day for five consecutive days. At day 6, the antinociceptive effects of morphine were assessed using the hot plate test again.

The antinociceptive response was calculated as a percentage of the maximum possible effect (%MPE), as follows: $\%MPE=[(T_t-T_o)/(T_c-T_o)]\times100$, where $T_o$ and $T_t$ are the paw licking or jumping latencies measured before ($T_o$) and after ($T_t$) morphine injection and $T_c$ is the cut-off time. The total antinociceptive effects were measured by plotting the %MPE (ordinate) against the time (h, abscissa) and calculating the area under the curve (AUC). These effects were expressed as %MPE.h.

### Reverse transcription and polymerase chain reaction (RT-PCR)

After the hot plate test, mice were sacrificed to collect brain samples for RT-PCR and autoradiography experiments. Immediately after removal from the skull, the entire cortex and the thalamus region were dissected on ice for use in RT-PCR. Total RNA was isolated from brain tissues using the RNeasy kit (Qiagen, Hilden, Germany), and 5 μg were reverse transcribed into cDNA using oligo $(dT)_{20}$ (SuperScript™ III First-Strand Synthesis System, Invitrogen, Carlsbad, CA, USA). Equal amounts of cDNA were used for PCR performed with Super PCR premix sapphire (Mbiotech, Seoul, Korea). PCR reactions (20 μL) were incubated at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min. The mouse *TRPV1* 320-bp product was amplified using the following primers: forward, 5'–GCATCTTCTACTTCAACTTCTTCGTC–3' and reverse, 5'–CCACATACTCCTTGCGATGGC–3'. The primers used to amplify β-actin (350-bp product, as a loading control) were as follows: sense, 5'–TGGAATCCTGTGGCTTCCATGAAAC–3'; and antisense, 5'–TAAAACGCAGCTCAGTAACAGTCCG–3'. The PCR products were separated on a 1.2% agarose gel containing ethidium bromide by electrophoresis (Sigma–Aldrich, St. Louis, MO, USA). Specific bands were quantified by densitometry using the Image Gauge software version 4.0 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

### Binding of [$^3$H]RTX to TRPV1

After being carefully removed from the skull, mouse brains were frozen on dry ice for 5 min. Brains were cut into 20-μm coronal sections at –19°C using a cryostat (Leica, Wetzlar, Germany) and were immediately mounted onto 1.5% gelatin-coated slides (Fisherbrand SuperFrost®/plus, Fisher Scientific, Pittsburgh, PA, USA), dried for 60 min at room temperature, and then stored at –80°C until use.

An autoradiography assay for TRPV1 was performed using [$^3$H]RTX as reported previously (Roberts *et al*., 2004) with slight modifications. In brief, sections were allowed to equilibrate at room temperature and were then incubated in assay buffer consisting of 10 mM HEPES, 5 mM KCl, 5.8 mM NaCl, 0.75 mM $MgCl_2$, 320 mM sucrose, and 1 mg/mL BSA (pH 7.4) containing 1 nM [$^3$H]RTX for 60 min at room temperature. Non-specific binding was determined by the addition of 1 μM unlabeled RTX to a parallel series of sections. After incubation, sections were washed 4 times with 20 mM Tris-HCl with 0.1 % BSA) for 10 min at 4°C and once with distilled water at 4°C to remove the buffer salts. The sections were allowed to air dry before being exposed to Kodak BioMax MR Film (Eastman Kodak Co., Rochester, NY, USA) along with [$^3$H]Autoradiographic Microscales™ (Amersham Bio-Science, NJ, USA) for six weeks.

Autoradiography films were developed at room temperature for 1 min and then fixed for 2 min. Quantification was performed using the Molecular Dynamic Image Quant software version 3.3 (Molecular Dynamics, Sunnyvale, CA, USA). Specific regions of interest were defined using the mouse brain atlas (Paxinos and Franklin, 2004). Standard curves for [$^3$H]Microscales™ were used to convert the optical density of each region into fmol/mg of wet brain tissue. Mean densities were determined from at least two sections per region per mouse.

**Table 1.** Percentage of the maximum possible effect (%MPE) of acute and chronic treatment of morphine over time

| Drug | Day 0 | | | | Day 6 | | | |
|---|---|---|---|---|---|---|---|---|
| | %MPE measured at the indicated time points | | | | %MPE measured at the indicated time points | | | |
| | 30 min | 60 min | 90 min | 120 min | 30 min | 60 min | 90 min | 120 min |
| SAL | 10.3 ± 3.8 | 13.2 ± 5.7 | 8.1 ± 6.3 | 2.6 ± 1.5 | 9.4 ± 4.9 | 5.2 ± 3.6 | 8.2 ± 3.4 | 6.2 ± 2.5 |
| MOR | 66.4 ± 10.9 (***) | 36.0 ± 12.5 | 30.6 ± 12.4 | 14.6 ± 9.1 | 17.7 ± 6.3 | 16.8 ± 7.4 | 12.5 ± 5.3 | 7.3 ± 4.8 |

The hot plate test was performed at day 0 (acute treatment) and day 6 (chronic treatment) at 30, 60, 90, and 120 min after morphine injection. The results are presented as the mean ± SEM (n=10–11). ***$p$<0.001 vs. the time-matched saline group at day 0, two-way repeated-measures ANOVA followed by Bonferroni post-hoc test. SAL, saline; MOR, morphine.

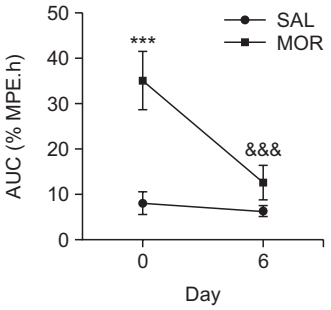


**Fig. 1.** Repeated morphine administration induced tolerance to its antinociceptive effects. Total antinociceptive effects were measured by calculating the area under the curve (AUC) in a plot of the percentage of the maximum possible effect (%MPE, ordinate) vs time (h, abscissa) and were expressed as %MPE.h. The results are presented as mean ± SEM (n=10–11). ***$p$<0.001 vs. the saline group at day 0; $^{\&\&\&}p$<0.001 vs the morphine group at day 0.

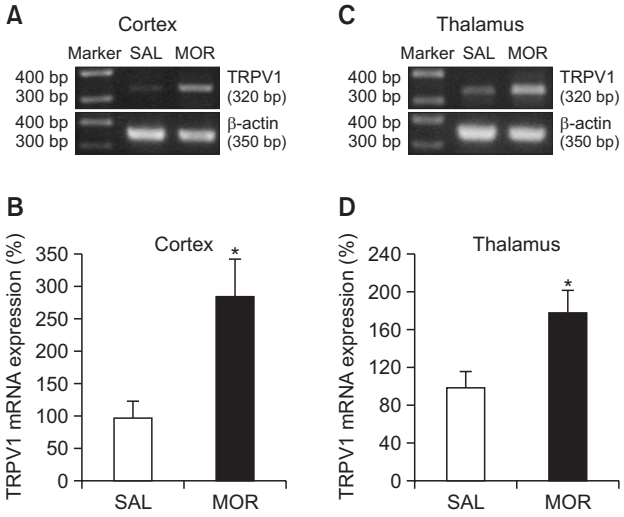


**Fig. 2.** Representative gel electrophoresis image (A, C) and densitometric analysis (B, D) of the TRPV1 mRNA levels in the cortex and thalamus. The results are presented as the mean ± SEM (n=3–4). *$p$<0.05 compared with the saline control group. SAL, saline; MOR, morphine.

### Statistical analyses

Data were expressed as the mean ± standard error of the mean (SEM). Experimental data were analyzed by two-tailed Student's t test or two-way repeated-measures analysis of the variance (ANOVA) followed by Bonferroni's multiple comparison tests using the Prism program version 8.0 (Graph Pad Software, Inc., San Diego, CA, USA). Differences were considered statistically significant at $p$<0.05.

## RESULTS

### Repeated morphine administration induced morphine tolerance in mice

The time-course analysis revealed that the %MPE significantly increased over time after acute morphine treatment (5 mg/kg) compared with that measured after saline injection, treatment: $F_{(1, 19)}$=13.26, $p$<0.01; time: $F_{(4, 19)}$=7.74, $p$<0.001; and treatment×time interaction: $F_{(4, 19)}$=3.87, $p$<0.01, two-way repeated-measures ANOVA (Table 1). In particular, at 30 min the %EPM measured in the morphine group was significantly increased compared to that determined for the saline control group ($p$<0.001, two-way repeated-measures ANOVA followed by Bonferroni post-hoc test, Table 1). After repeated morphine injections (10 mg/kg) for five consecutive days, 5 mg/kg of morphine did not induce any significant difference in the %MPE between the morphine and saline groups ($p$>0.05, two-way repeated-measures ANOVA followed by Bonferroni post-hoc test, Table 1).

In addition, the analysis of the total antinociceptive effects (or AUC) in the morphine and saline groups revealed a significant treatment and day interaction, treatment: $F_{(1, 19)}$=13.69, $p$<0.01; day: $F_{(1, 19)}$=10.05, $p$<0.01; and treatment×day interaction: $F_{(1, 19)}$=7.38, $p$<0.05; two-way repeated-measures ANOVA. The total antinociception was significantly higher in morphine-treated mice than that measured for saline-treated mice at day 0 but not at day 6 ($p$<0.001 at day 0 and $p$>0.05 at day 6, two-way repeated-measures ANOVA followed by Bonferroni post-hoc test, Fig. 1). Moreover, the antinociceptive effects of morphine were significantly diminished at day 6 compared with the effects measured in the morphine-treated mice at day 0 ($p$<0.001, two-way repeated-measures ANOVA followed by Bonferroni post-hoc test, Fig. 1).

### Upregulation of the TRPV1 mRNA in the cortex and thalamus of mice with morphine tolerance

To evaluate *TRPV1* expression in morphine-tolerant mice, an RT-PCR experiment was performed on brain samples from morphine-tolerant mice at the end of the behavioral test. RT-PCR data showed that the *TRPV1* mRNA levels were increased in the cortex (285.7% of control levels, $p$<0.05, Fig.

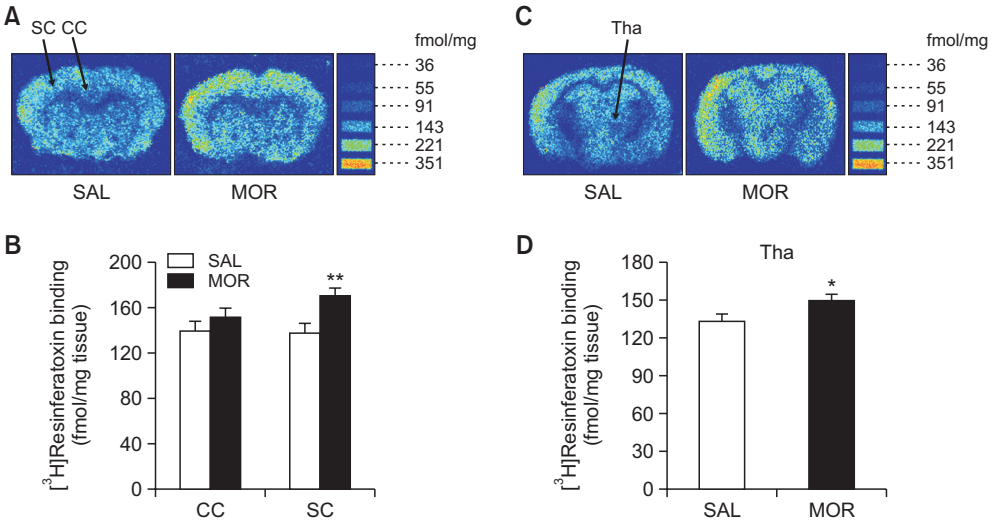


**Fig. 3.** Representative autoradiogram (A, C) and quantitative autoradiographic analysis (B, D) of [$^3$H]resiniferatoxin binding in the cingulate cortex, sensory cortex, and thalamus. The results are expressed as the mean ± SEM (n=10–11). **$p$<0.01 and *$p$<0.05 compared with the saline control group. SAL, saline; MOR, morphine; CC, cingulate cortex; SC, sensory cortex; Tha, thalamus.

2A, 2B) and thalamus (178.6% of control levels, $p$<0.05, Fig. 2C, 2D) of morphine-treated mice compared with the levels detected in the control group.

### Increased binding of [$^3$H]RTX in the sensory cortex and thalamus of mice after repeated exposure to morphine

To examine whether morphine treatment affects the binding site of TRPV1 in the brain of mice with morphine tolerance, an autoradiographic analysis was performed using [$^3$H]RTX. Binding of [$^3$H]RTX was observed in three brain regions: the cingulate cortex, sensory cortex, and thalamus. The autoradiographic data revealed that this binding was significantly increased in the sensory cortex ($p$<0.01, Fig. 3A, 3B) and thalamus ($p$<0.05, Fig. 3C, 3D) but not in the cingulate cortex (Fig. 3A, 3B) of mice after repeated treatment with morphine.

## DISCUSSION

"Pain is unpleasant sensory or emotional experience associated with actual or potential tissue damage or described in terms of such damage. Pain is always subjective" (Merskey, 1994). The pathways transmitting noxious information to the brain are complex. In general, painful information is transmitted from peripheral sites to the spinal cord through first-order neurons and then from the spinal cord to the thalamus through second-order neurons. Finally, thalamic neurons send painful information to the sensory cortex (Stucky *et al*., 2001). Even though pain pathways are complex, all noxious information must go through and be processed by the thalamus before reaching the sensory cortex (Dostrovsky, 2000). Therefore, the thalamus and sensory cortex are important components of the pain pathway.

In the present study, we quantified *TRPV1* mRNA expression and binding sites in the brain of mice with morphine tolerance. Our data revealed that repeated morphine treatments increased *TRPV1* expression and binding sites in pain-related brain regions, specifically the sensory cortex and thalamus.

Here, the treatment schedule followed the tolerance protocol published in our previous study using the same mouse species (Nguyen *et al*., 2010). This schedule induces significant tolerance to morphine analgesia in naïve ICR mice. In that study, we found that acute treatment with a TRPV1 antagonist potentiated the antinociceptive effects of morphine, and that repeated co-treatment with the TRPV1 antagonist significantly prevented the development of tolerance to, and the physical dependence on morphine (Nguyen *et al*., 2010). Repeated daily morphine treatment (10 mg/kg, s.c.) has also been reported to favor the development of a tolerance in a formalin-induced inflammation pain model in ddY mice after four days (Rahman *et al*., 1994). Our behavioral data are consistent with those previous studies and show a significant tolerance to morphine analgesia in mice after daily morphine injections (10 mg/kg, s.c.) for five days.

Repeated morphine administration induces tolerance in animals and humans regardless of the administration route (Cichewicz and Welch, 2003; Younger *et al*., 2008). Tolerance to morphine analgesia develops at peripheral, spinal, and supraspinal sites (Leavens *et al*., 1982; Madrid *et al*., 1987; Kolesnikov *et al*., 1996; Ness and Follett, 1998; Kest and Hopkins, 2001; Bao *et al*., 2015) through complex mechanisms that are not fully understood. Numerous studies have indicated that the activation of pronociceptive chemicals contributes to morphine tolerance. Hutchinson *et al*. (2008) reported that chronic intrathecal morphine administration increases the release and expression of spinal chemokines and cytokines, which reverse the analgesic effects of morphine. Elevated nociceptive neurotransmitter levels, which undermined morphine analgesic effect, after sustained morphine exposure of the spinal cord and DRGs have been demonstrated both *in vivo* (Gardell *et al*., 2002; King *et al*., 2005) and *in vitro* (Ma *et al*., 2000; Yue *et al*., 2008). Recently, Chen *et al*. (2008) found that chronic morphine administration increases TRPV1 expression in the sciatic nerve, DRGs, and spinal cord through a mitogen-activated protein kinase pathway that contributes to morphine tolerance. The present data provide new information

indicating that repeated morphine treatment increases TRPV1 expression at supraspinal sites of the pain pathway, and that these increased levels might be involved in morphine tolerance. Central TRPV1s, albeit less abundant, play an important role in pain-related behaviors since TRPV1 antagonists that have greater central nervous system penetration block more efficiently some pain states (Cui *et al*., 2006). In the development of tolerance to morphine, TRPV1 is not only upregulated at peripheral and spinal sites (Chen *et al*., 2008) but also in supraspinal sites demonstrated here.

Although the role of TRPV1 in the brain is not fully understood, brain TRPV1 might be involved in pain-related behaviors and in psychological disorders induced by morphine. TRPV1 blockade by its antagonist significantly reduces morphine-induced withdrawal symptoms in mice (Nguyen *et al*., 2010). TRPV1s in the nucleus accumbens and dorsal striatum play a role in morphine-induced place preference (Nguyen *et al*., 2014; Hong *et al*., 2017). Administration of TRPV1 antagonists into the nucleus accumbens significantly reduces the morphine self-administration behavior in rats (Ma *et al*., 2018). Microinjection of TRPV1 antagonists into the locus coeruleus significantly decreases the expression of some morphine withdrawal symptoms in rats (Fatemi *et al*., 2019). Together with these previous studies, our data suggest that brain TRPV1 is not only involved in the analgesic effects of morphine, but also in its psychological effects including addiction and dependence. However, the mechanisms underlying the involvement of TRPV1 and morphine effects remain to be clarified.

Morphine produces therapeutic and adverse effects, including the development of tolerance and respiratory depression, through the activation of μ-opioid receptors, which transduce two parallel and dissociable signaling cascades: G-protein signaling pathways and β-arrestin-mediated signaling pathways (Whalen *et al*., 2011). G-protein signaling pathways induce the analgesic effect via coupling to Gαi subunits, which inhibit the production of cyclic adenosine monophosphate (cAMP) (Pasternak and Pan, 2013). Controversially, the β-arrestin signaling pathway can be involved in the adverse effects of morphine, especially in the tolerance to antinociception (Colvin *et al*., 2019). The μ-opioid receptor and TRPV1 were found to co-localize in DRG neurons and the spinal cord (Chen and Pan, 2006; Chen *et al*., 2008). Interactions between μ-opioid receptors and TRPV1 were reported both in *in vitro* and *in vivo* studies. In addition, μ-opioid receptors and TRPV1 reciprocally regulated each other via the β-arrestin 2 signaling pathway in HEK293 cells (Melkes *et al*., 2020). The activation of the μ-opioid receptor induces sensitization of TRPV1 by sequestering β-arrestin-2 away from TRPV1, thus reducing the TRPV1/β-arrestin-2 association and increasing TRPV1 activity in sensory neurons (Rowan *et al*., 2014). In turn, the activation of TRPV1 induces the translocation of G-protein-coupled receptor kinase 5 to the nucleus, thereby blocking μ-opioid receptor phosphorylation and preventing μ-opioid receptor internalization from the cell membrane via β-arrestin signaling (Scherer *et al*., 2017). In contrast, blocking TRPV1 receptors using TRPV1 antagonists significantly attenuated morphine tolerance in mice (Nguyen *et al*., 2010) and rats (Chen *et al*., 2008). Moreover, deletion of TRPV1-expressing sensory neurons by intrathecal administration of resiniferatoxin (RTX) prevented the development of morphine tolerance in rats (Chen *et al*., 2007). Taken together, these data showed that the activation and blockade of TRPV1 may reduce morphine tolerance via different mechanisms; the activation of TRPV1 prevents μ-opioid receptor desensitization, thereby maintaining sufficient function of μ-opioid receptors, whereas blockade of TRPV1 diminishes morphine tolerance, probably by neutralizing TRPV1 sensitization (Bao *et al*., 2015). Therefore, TRPV1 may be an important target for improving the therapeutic profile of morphine.

In conclusion, we have reported for the first time that repeated morphine treatment induced the upregulation of TRPV1 in the sensory cortex and thalamus of morphine-tolerant mice. Our data suggest that brain TRPV1 might also be involved in the supraspinal mechanisms of morphine tolerance.

## CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest regarding the conduct and reporting of research.

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