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Transient Receptor Potential Vanilloid Type 1 Channel May Modulate Opioid Reward

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Transient receptor potential vanilloid type 1 (TRPV1), a nonselective cation channel, is a well-known pain-related receptor. TRPV1 involvement in morphine-induced antinociception, tolerance, and withdrawal symptoms has been previously reported. Emerging evidence indicates that TRPV1 may be related to both the cellular and behavioral effects of addictive drugs. In the present study, we investigated the role of TRPV1 in morphine reward using the conditioned place preference (CPP) paradigm in mice. Repeated morphine treatments upregulated TRPV1 expression in the dorsal striatum (DSt). Treatment with a TRPV1 agonist potentiated morphine reward, and pretreatment with TRPV1 antagonists attenuated these effects. Microinjection of a selective TRPV1 antagonist into the DSt significantly inhibited morphine-CPP. In addition, treatment with a TRPV1 antagonist suppressed morphine-induced increases in μ-opioid receptor binding, adenylyl cyclase 1 (AC1), p38 mitogen-activated protein kinase (p38 MAPK), and nuclear factor kappa B (NF-κB) expression in the DSt. Administering a p38 inhibitor not only prevented morphine-CPP, but also prevented morphine-induced NF-κB and TRPV1 activation in the DSt. Furthermore, injecting an NF-κB inhibitor significantly blocked morphine-CPP. Our findings suggest that TRPV1 in the DSt contribute to morphine reward via AC1, p38 MAPK, and NF-κB. Brain TRPV1 may serve as a novel therapeutic target to treat morphine-addictive disorders.

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INTRODUCTION

The opiate drug morphine has a long history of use both for medical and recreational purposes. Even though intense efforts have been focused on the issue for several centuries, it remains difficult to separate the therapeutic effects of the drug from its adverse side effects. One of the major drawbacks of repeated opiate use is the development of drug addiction because of the powerful rewarding properties of opiates. Drug addiction is a disorder characterized by compulsive drug consumption despite adverse health, economic, and social consequences, and is plagued by chronic relapse (Koob and Volkow, 2010). Even though the exact molecular and cellular mechanisms underlying morphine addiction are not yet fully understood, it is thought that long-term neural adaptations in specific brain reward regions have a crucial role (Nestler and Aghajanian, 1997). Repeated morphine exposure alters gene expression and subsequently induces stable changes in protein components (Ammon-Treiber and Hollt, 2005). There is mounting evidence suggesting that the alteration of protein profiles in the brain after repeated morphine exposure underlie morphine addiction (Bierczynska-Krzysik *et al*, 2006; Kim *et al*, 2005; Li *et al*, 2006a). Numerous proteins that change their expression pattern following morphine treatment are listed in these publications, but particular candidate proteins for morphine reward-related proteins have not been evaluated. The development of effective treatment for morphine addiction requires identifying a specific protein that has a key role in morphine-rewarding effects.

Transient receptor potential vanilloid type 1 (TRPV1) is a nonselective cation channel activated by endogenous lipids, capsaicin, heat, and low pH (Tominaga *et al*, 1998). Although TRPV1 expression in the brain is well documented (Cristino *et al*, 2006; Starowicz *et al*, 2008; Toth *et al*, 2005), its roles in health and disease are just beginning to be explored. Genetic association studies have revealed that the *TRPV1* gene is involved in cannabinoid-addictive disorders (Agrawal and Lynskey, 2009). Growing evidence suggests that TRPV1 may be involved in the neuronal and behavioral adaptations induced by addictive drugs such as drug consumption, drug seeking, anxiety, and depression. Deleting the *TRPV1* gene in mice can alter ethanol consumption (Blednov and Harris, 2009) and diminish anxiety and conditioned fear (Marsch *et al*, 2007); blocking TRPV1 also suppresses cocaine-seeking behavior in the reinstatement phase (Adamczyk *et al*, 2012). Intraprefrontal

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cortex or intraperitoneal (i.p.) administration of TRPV1 antagonist induces anxiolytic-like effects (Aguiar *et al*, 2009; Kasckow *et al*, 2004), and in preclinical models of depression-like behaviors induced by nicotine or immobilization stress, TRPV1 agonists show antidepressant-like effects in both the forced swimming and tail suspension tests (Hayase, 2011). Moreover, upregulation of TRPV1 in the dorsal root ganglion, spinal cord, and sciatic nerve through mitogen-activated protein kinase pathways after chronic morphine treatment contributed to morphine tolerance in rats (Chen *et al*, 2008). Recently, we reported that blocking TRPV1 with a TRPV1 antagonist, capsazepine, attenuated morphine tolerance and withdrawal symptoms in mice (Nguyen *et al*, 2010). Taken together, these data imply that TRPV1 may be a molecular target in morphine reward.

In the present study, we therefore sought to determine whether TRPV1 expression in the brain is altered by repeated morphine administration, and to assess the role of TRPV1 in morphine reward in mice. Furthermore, we studied possible mechanisms to explain how TRPV1 contributes to morphine reward.

MATERIALS AND METHODS

Animals

Four-week-old male Institute for Cancer Research mice (ICR (CD1) mice, 22–25 g, Dae Han Biolink, Eumseong, Korea) were used in the present study. Mice were acclimatized in an animal room under a 12-h light/dark cycle at $22 \pm 2\,^{\circ}C$ and fed ad libitum. All animal care procedures were conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the study protocol was approved by the Institutional Animal Care and Use Committee of Sungkyunkwan University.

Chemicals

Morphine hydrochloride (Macfarlan Smith, Edinburgh, UK) and pyrollidine dithiocarbamate (PDTC; Sigma-Aldrich, St Louis, MO, USA) were dissolved in physiological saline. Capsazepine, capsaicin, SB203580 hydrochloride, and SB366791 (Tocris Cookson, Bristol, UK) were dissolved in physiological saline containing 10% dimethyl sulfoxide and 10% Tween 80 (Sigma-Aldrich).

Guide Cannula Implantation and IntraDorsal Striatum Microinfusion

Mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and a guide cannula (9 mm, 24 gauge) was implanted laterally into the dorsal striatum (DSt). The coordinates were as follows: + 0.86 mm A/P, + 1.5 mm M/L, and − 2.7 mm D/V according to the atlas of Paxinos and Franklin (2004). Infusions were performed at a rate of 1 μl/min/site for 1 min.

Conditioned Place Preference Procedure

Conditioned place preference (CPP) was performed in an apparatus consisting of two plexiglass compartments ($15 \times 15 \times 15$ cm): one white with a textured floor and one black with a smooth floor. In the preconditioning phase, mice were habituated in the apparatus (day 1) and the displacement of the mice was recorded for 20 min on day 2. In the conditioning phase (days 3–10), mice were confined to the less preferred compartment for 60 min after morphine injection (5 mg/kg, subcutaneously (s.c.)) on days 3, 5, 7, and 9, or to the preferred compartment after saline injection on days 4, 6, 8, and 10. During the post-conditioning phase (day 11), the time that the mice spent in each compartment was recorded for 20 min. CPP scores were expressed as the differences between the pretesting and post-testing times that the mice spent in the drug-paired compartment.

On day 11, after finishing the behavior test, 6-week-old mice were killed by decapitation. Mouse brains were carefully removed, frozen in dry ice, and stored at $-80\,^{\circ}C$ for further study, including autoradiography, real-time RT-PCR, and western blot.

Quantitative Receptor Autoradiography

Tissue preparation. Mouse brains were cut into 20-μm coronal sections at $-19\,^{\circ}C$ using a cryostat (Leica, Wetzlar, Germany). Sections were immediately mounted on 1.5% gelatin-coated slides (Fisher Scientific, PA, USA) and stored at $-80\,^{\circ}C$ until use.

TRPV1 receptor binding. Brain sections were incubated in assay buffer containing 1 nM [^{3}H]resiniferatoxin (38.31 Ci/mmol, PerkinElmer, Boston, MA, USA) for 60 min at room temperature. Nonspecific binding was controlled by adding 1 μM-unlabeled resiniferatoxin to a parallel series of sections. The sections were allowed to air dry and were exposed to Kodak BioMax MR Film (Eastman Kodak, Rochester, NY, USA) along with [^{3}H]autoradiographic microscales (Amersham Biosciences, NJ, USA) at $4\,^{\circ}C$ for 6 weeks.

μ-Opioid receptor binding. Brain sections were incubated in buffer containing 4 nM [^{3}H]DAMGO (56.8 Ci/mmol, PerkinElmer) at room temperature for 60 min. Slides were exposed to Kodak BioMax MR film (Eastman Kodak) along with [^{3}H]autoradiographic microscales (Amersham Biosciences) at $4\,^{\circ}C$ for 3 weeks.

Autoradiography films were developed and quantified using Molecular Dynamic Image Quant software version 3.3.

Real-Time RT-PCR

Tissue preparation. Coronal brain sections (Bregma + 1.7 to + 0.74 mm) according to the mouse brain atlas (Paxinos and Franklin, 2004) were collected. The DSt regions were punched bilaterally using a Harris Uni-Core 1.20 (Ted Pella, Redding, CA, USA). These brain tissues were used for real-time RT-PCR and western blot assays.

Real-time RT-PCR. Total RNA was isolated from brain tissues using the RNeasy microkit (Qiagen, Valencia, CA, USA) and quantified spectrophotometrically. Real-time PR-PCR was performed using an Invitrogen kit with TRPV1 primers (forward: 5′-AGCCATGCTCAATCTGCAC-3′ and reverse: 5′-TGCTGTCTGGCCCTTGTAG-3′); and β-actin primers (forward: 5′-AGAGGGAAATCGTGCGTGAC-3′ and reverse: 5′-CAATAGTGATGACCTGGCCT-3′). The

comparative critical threshold, $\Delta\Delta C_t$, was calculated using Corbett Research software version 1.7.75. The relative TRPV1 mRNA expression of each sample was the mean of triplicate measurements.

Western Blot

Tissues were homogenized and then were centrifuged for 20 min at 15 000 r.p.m. at 4 °C to collect the supernatant. Supernatants (50 μg) were separated by 6% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were incubated with primary antibodies and then with secondary antibodies. Binding sites were visualized on Kodak BioMax MR Film (Eastman Kodak) using a Western Lightning Detection Kit (PerkinElmer Life Science). Specific bands were quantified by densitometric analysis using Image Gauge software version 4.0. Data were calculated as a relative ratio to the control.

Immunohistochemistry

Tissue preparation. After CPP, mice were intracardially perfused with a solution containing 4% paraformaldehyde. Brains were post fixed in 4% paraformaldehyde and then placed in 30% sucrose, after which they were frozen and sectioned coronally at 40 μm using a cryostat (Leica).

Immunohistochemistry. Brain sections were incubated with the primary antibodies. For immunoperoxidase staining, biotinylated secondary antibody (7 μg/ml; Vector Laboratories) was applied, followed by incubation with an avidin–biotin horseradish peroxidase complex (Vector Laboratories). The peroxidase label was detected by incubating in 3,3′-4,4′-diaminobenzidine (Sigma). For double-fluorescent staining, a mixture of Alexa Fluor 488 anti-rabbit and Alexa Fluor 568 anti-guinea pig IgG antibodies (1:1000; Molecular Probes) was applied. Images were taken using a DP digital microscope camera (Olympus Optical, Tokyo, Japan) connected to a microscope (BX 51, Olympus, Tokyo, Japan).

p-p38, pNF-κB (p-nuclear factor kappa B), and adenylyl cyclase type 1 and 8 (AC1 and AC8) IR cells were manually counted in a fixed box size of $150 \times 150\,\mu m^2$ in the center of the DSt by an observer blinded to the experimental groups. Data were represented as a relative ratio to the control (%). Details on methods used in this study are provided in the Supplementary Information.

Statistical Analyses

The data were expressed as mean ± SEM. The experimental data were analyzed by one-way analysis of variance followed by the Newman–Keuls *post hoc* test or Student's *t*-test using Prism version 5.0 (Graph Pad Software). A value of $P<0.05$ was considered statistically significant.

RESULTS

Morphine-Upregulated TRPV1 Expression in the DSt

In order to determine the effects of morphine on TRPV1 expression, we administrated morphine (5 mg/kg, s.c., every other day for 4 days) following the CPP schedule. Morphine treatment increased significantly the TRPV1 mRNA level in the DSt in the morphine group (3.64 ± 1.02 *vs* 1.00 ± 0.30, $t(12) = 2.47$, $P = 0.029$; Figure 1b). In addition, TRPV1 protein levels in the DSt were also higher in the morphine group (2.30 ± 0.13 *vs* 1.00 ± 0.05, $t(4) = 9.88$, $P<0.001$; Figure 1c and d). Moreover, [^{3}H]resiniferatoxin binding in the DSt increased in the morphine group compared with the control group (157.9 ± 5.12 *vs* 133.4 ± 7.75 fmol/mg tissue, $t(24) = 2.71$, $P = 0.012$; Figure 1e and f).

Effects of a TRPV1 Agonist and Antagonists and on Morphine-CPP

On the conditioning days, mice were received i.p. injection of a TRPV1 agonist, capsaicin, or of TRPV1 antagonists 30 min before administering s.c. morphine. Pretreatment with TRPV1 antagonists significantly suppressed morphine-CPP. Capsazepine (2.5 mg/kg, i.p.) significantly reduced morphine-CPP ($F(4,47) = 4.42$, $P = 0.004$; *post hoc* test, $P<0.05$; Figure 2a). SB366791 pretreatment (37.5, 75, and 150 μg/kg, i.p.) reduced CPP scores in a dose-dependent manner compared with vehicle/morphine ($F(5,64) = 2.83$, $P = 0.022$). At 150 μg/kg, SB366791 significantly blocked morphine-CPP (*post hoc* test, $P<0.05$; Figure 2b). In contrast, pretreatment with capsaicin significantly enhanced morphine-CPP ($F(4,39) = 4.33$, $P = 0.033$). Alone, a low dose of morphine (1.5 mg/kg, s.c.) did not significantly induce CPP. However, when co-treated with capsaicin (200 μg/kg, i.p.), low-dose morphine (1.5 mg/kg, s.c.) remarkably increased CPP score compared with vehicle (*post hoc* test, $P<0.01$) or morphine control (*post hoc* test, $P<0.05$, Figure 2c).

To verify that the effects of capsaicin on morphine-CPP were mediated by TRPV1 receptor activation and not nonspecific activation of other receptors, we examined the effects of capsaicin on morphine-CPP in the presence of SB366791. First, SB366791 was injected, 15 min later capsaicin was injected. and 30 min later morphine was injected. As shown in Figure 2d, capsaicin (200 μg/kg, i.p.) significantly potentiated morphine-CPP compared with the vehicle or morphine control groups ($F_{(4,58)} = 3.82$, $P = 0.008$). These effects were antagonized by SB366791 (*post hoc* test, $P<0.05$). Administration of capsaicin, capsazepine, and SB366791 did not produce any significant differences in CPP score compared with the vehicle control groups.

As systemic administration of a TRPV1 agonist and antagonists significantly affected the CPP score of morphine and repeated morphine treatment upregulated TRPV1 in the DSt, striatum TRPV1 may have a role in morphine reward. To validate this hypothesis, a selective TRPV1 antagonist, SB366791, was injected laterally into the DSt 30 min before morphine injection on each conditioning day. As shown in Figure 2e and f, SB366791 (0.2 ng/site) significantly attenuated morphine-induced CPP ($F(2,24) = 5.84$, $P = 0.009$; *post hoc* test, $P<0.05$).

Effects of a TRPV1 Antagonist on μ-Opioid Receptor Binding in the DSt of Morphine-CPP Mice

To examine whether TRPV1 antagonist affects the binding site of μ-opioid receptors in the DSt, autoradiographic analysis was performed using [^{3}H]DAMGO. Autoradiographic data showed that repeated morphine treatments significantly increased [^{3}H]DAMGO binding in the DSt ($F(2,26) = 5.37$,

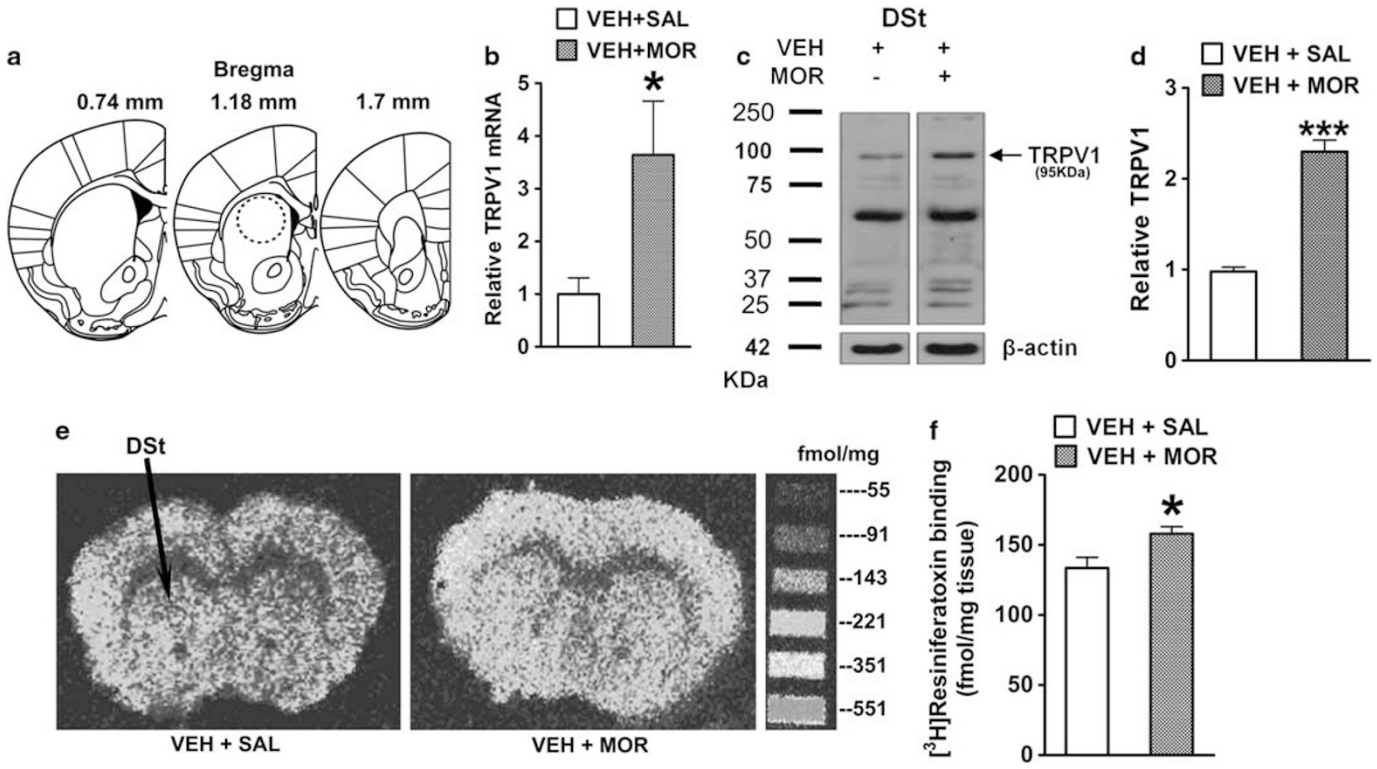

Figure 1 Morphine-upregulated transient receptor potential vanilloid type 1 (TRPV1) expression in the dorsal striatum (DSt). (a) The locations of punched samples. (b) Quantitative analysis of TRPV1 mRNA levels ($n = 7$/group). (c, d) Representative images and quantitative analysis of TRPV1 levels ($n = 3$/group). (e, f) Representative autoradiograph and quantitative analysis of [^{3}H]resiniferatoxin binding ($n = 12$–14/group). ***$P < 0.001$, *$P < 0.05$ *vs* the control group. MOR, morphine (5 mg/kg); SAL, saline; VEH, vehicle.

$P < 0.011$) of morphine-CPP mice compared with the vehicle control group (Figure 3). These increases were diminished by capsazepine pretreatment (*post hoc* test, $P < 0.05$).

A TRPV1 Antagonist Suppresses Morphine-Induced Increases in AC1 Expression in the DSt

Superactivation of AC1 and AC8 is known to have a role in morphine dependence (AC1 and AC8) (Lane-Ladd *et al*, 1997). Knocking out both *AC1* and *AC8* genes significantly reduces morphine-CPP, suggesting that AC1 and/or AC8 are necessary for morphine-CPP (Li *et al*, 2006b). Thus, we determined levels of AC1 and AC8 in CPP samples treated with SB366791. Immunohistochemistry data revealed that the number of AC1 IR cells was increased in the DSt ($F(2,15) = 12.56$, $P < 0.001$) of morphine-CPP mice (Figure 4a and b). Pretreatment with SB366791 significantly blocked morphine-induced increases in AC1 in the DSt (*post hoc* test, $P < 0.01$; Figure 4a and b). There were no significant changes in the number of AC8 IR cells in the DSt ($F(2,15) = 0.67$, NS; Figure 4c) among the saline, morphine, and SB366791-morphine groups. Western blot data also indicated that SB366791 significantly reduced morphine-induced increases in AC1 in the DSt ($F(2,6) = 111.2$, $P < 0.001$; *post hoc* test $P < 0.01$; Figure 4d and e).

TRPV1 Antagonists Suppress Morphine-Activated p38/NF-κB in the DSt

Previously, it was reported that the p38/ NF-κB pathway is involved in morphine reward in rats (Zhang *et al*, 2011). To verify this finding, we studied the effects of p38 inhibitor SB203580 and NF-κB inhibitor PDTC in morphine-CPP in mice. The CPP data revealed that SB203580 (0.125, 0.25, and 0.5 mg/kg, i.p.) prevented morphine-CPP in a dose-dependent manner ($F_{(5,67)} = 3.85$, $P = 0.004$). At 0.5 mg/kg, SB203580 significantly blocked morphine-CPP (*post hoc* test, $P < 0.05$; Figure 5e). No significant difference in CPP scores was detected between the SB203580-saline and vehicle-saline groups. Similarly, PDTC (5, 25, and 50 mg/kg, i.p.) prevented morphine-CPP in a dose-dependent manner ($F_{(5,53)} = 3.24$, $P = 0.013$; Figure 5j). At 50 mg/kg, PDTC significantly blocked morphine-CPP (*post hoc* test, $P < 0.05$). No significant difference in CPP scores was detected between the PDTC–saline and saline–saline groups. Our data suggest that p38 and NF-κB activation are necessary for morphine reward in mice.

To investigate whether the effects of TRPV1 antagonist SB366791 on morphine reward are related to p38 and NF-κB, the levels of p-p38 and pNF-κB were assessed in CPP samples treated with SB366791. The number of cells expressing p-p38 (Figure 5a and b) and pNF-κB (Figure 5f and g) in the DSt increased significantly in the morphine control group ($F_{(2,15)} = 5.33$, $P = 0.018$; and $F_{(3,20)} = 5.91$, $P = 0.005$, respectively). These increases were attenuated by SB366791 (*post hoc* test, $P < 0.05$ and $P < 0.05$, respectively). Consistent with immunohistochemistry data, western blot data indicated that SB366791 treatment significantly decreased morphine-induced increases in p-p38 ($F(2,6) = 110.0$, $P < 0.001$; *post hoc* test $P < 0.001$; Figure 5c and d) and pNF-κB ($F(3,8) = 316.3$, $P < 0.001$; *post hoc* test, $P < 0.001$; Figure 5h and i) in the DSt. In addition, a p38 inhibitor,

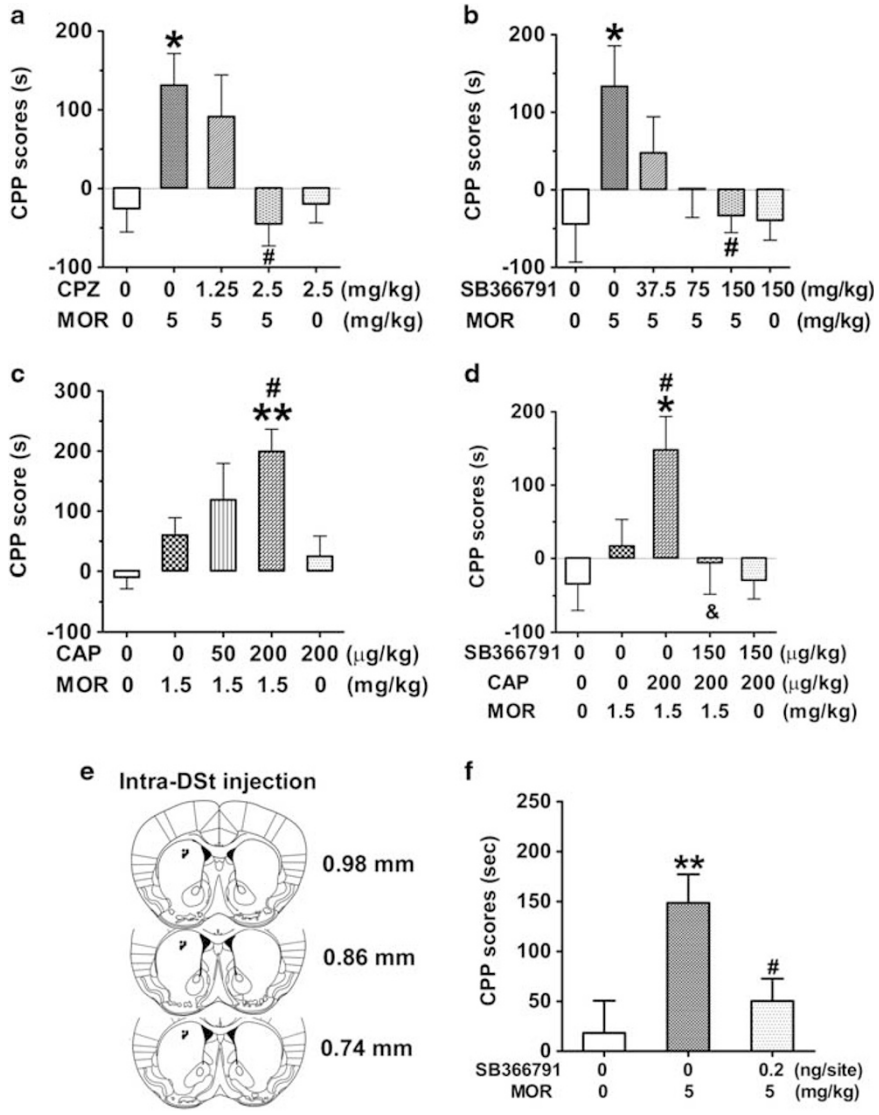

Figure 2 Effects of a transient receptor potential vanilloid type 1 (TRPV1) agonist and antagonists on morphine-conditioned place preference (CPP). Capsazepine (a) and SB366791 (b) prevented morphine-CPP in mice ($^{*}P<0.05$ *vs* VEH + SAL; $^{\#}P<0.05$ *vs* VEH + MOR). (c) Capsaicin-enhanced morphine-CPP in mice ($^{**}P<0.01$ *vs* VEH + SAL; $^{\#}P<0.05$ *vs* VEH + MOR). (d) SB366791 antagonizes the effects of capsaicin on morphine-CPP in mice ($^{*}P<0.05$ *vs* VEH + VEH + SAL; $^{\#}P<0.05$ *vs* VEH + VEH + MOR; $^{\&}P<0.05$ *vs* VEH + CAP + MOR). (e) The location of microinjection into the dorsal striatum (DSt). (f) Microinjection of a TRPV1 antagonist into the DSt significantly prevented morphine-CPP. $n=8$–13/group. $^{**}P<0.01$ *vs* VEH + SAL; $^{\#}P<0.05$ *vs* VEH + MOR. CAP, capsaicin; CPZ, capsazepine; MOR, morphine; SAL, saline; VEH, vehicle.

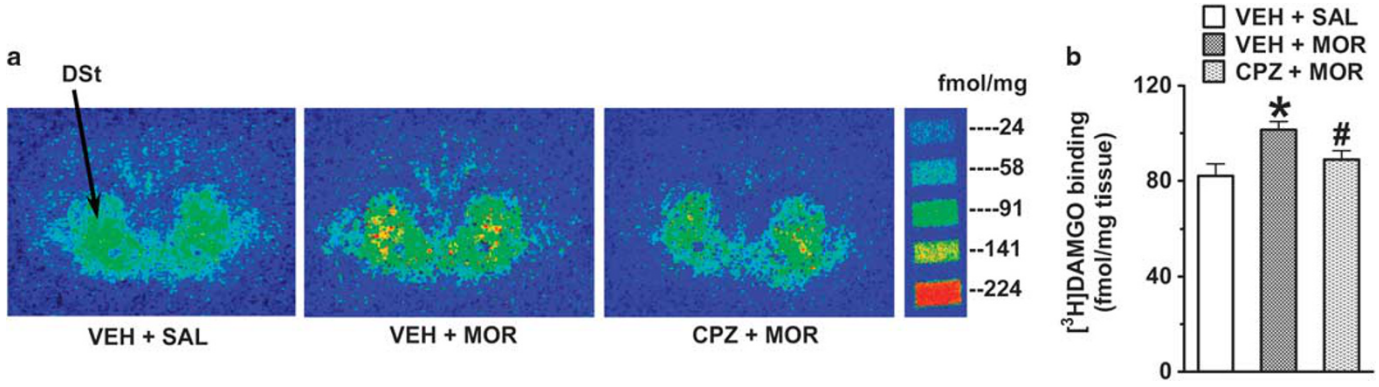

Figure 3 The effects of a transient receptor potential vanilloid type 1 (TRPV1) antagonist on [^{3}H]DAMGO binding in the dorsal striatum (DSt) of morphine-conditioned place preference (CPP) mice. Representative autoradiogram (a) and quantitative analysis (b) of [^{3}H]DAMGO binding in mouse brains ($n=9$–10/group). $^{*}P<0.05$ *vs* VEH + SAL; $^{\#}P<0.05$ *vs* VEH + MOR. CPZ, capsazepine (2.5 mg/kg); MOR, morphine (5 mg/kg); SAL, saline; VEH, vehicle.

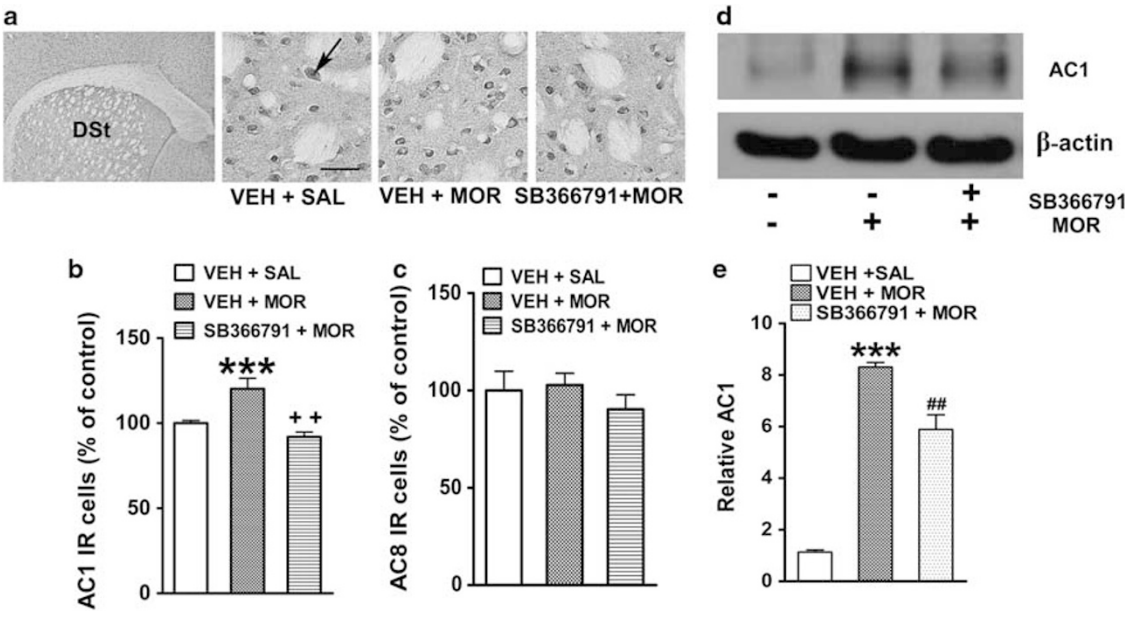

Figure 4 Effects of a transient receptor potential vanilloid type 1 (TRPV1) antagonist on adenylyl cyclase type 1 and 8 (AC1 and AC8) expression. (a) Photomicrographs of AC1 IR cells in the dorsal striatum (DSt). The arrow indicates an AC1 IR cell; scale bars, 40 μm. (b, c) Quantitative analysis of the number of AC1 and AC8 IR cells ($n = 6$/group). ***$P < 0.001$ *vs* VEH + SAL; $^{++}P < 0.01$ *vs* VEH + MOR. (d, e) Representative images and quantitative analysis of AC1 levels in the DSt ($n = 3$/group). ***$P < 0.001$ *vs* VEH + SAL; $^{\#\#}P < 0.01$ *vs* VEH + MOR. MOR, morphine (5 mg/kg); SB366791 (150 μg/kg); SAL, saline; VEH, vehicle.

SB203580, diminished the increase of pNF-κB in the DSt (*post hoc* test, $P < 0.01$; Figures 5f and g and $P < 0.001$; Figure 5h and i), confirming that p38 functions upstream of NF-κB in a signaling cascade involved in morphine reward.

DISCUSSION

In the present study, we found that repeated morphine administration upregulated TRPV1 expression in the DSt, having a role in morphine reward. Our data are consistent with the results of previous studies, which have reported that TRPV1 is widely expressed and functions in many brain structures including the DSt (Cristino *et al*, 2006; Maccarrone *et al*, 2008). However, a recent study suggested that TRPV1 expression was limited to the caudal hypothalamus and adjacent areas (Cavanaugh *et al*, 2011). In that study, TRPV1 expression was examined using reporter sequences inserted into the final exon of the *TRPV1* gene. However, their methods did not account for the possibility of TRPV1 isoforms, such as splice variants, that do not include the final exon. In addition, the internal ribosome entry site in the reporter sequence may have been incompatible with certain brain cells, leading to false negatives. We used multiple techniques, namely RT-PCR, western blot analysis, and autoradiographic binding, to accurately confirm the presence of TRPV1 in the DSt.

Two previous studies have demonstrated that TRPV1 has a role in synaptic transmission and neuroplasticity in the striatum (Grueter *et al*, 2010; Maccarrone *et al*, 2008). TRPV1 is functional in synaptic structures and participates in forming neural networks and regulating vesicle recycling (Goswami *et al*, 2010). As a synaptic protein with a role in neuroplasticity, upregulation of TRPV1 in the DSt may modify synaptic functions and significantly contribute to morphine reward. Indeed, our behavioral data confirmed the critical contribution of TRPV1 in morphine reward. Blocking TRPV1 with different selective antagonists significantly suppressed morphine-CPP. In contrast, activation of TRPV1 with a selective agonist potentiated morphine-CPP. In addition, pretreatment with a TRPV1 antagonist reversed the effects of the TRPV1 agonist on morphine-CPP, confirming that the effects of TRPV1 antagonists and agonist on morphine-CPP were because of selective effects on TRPV1 and not off-target effects. As capsaicin by itself did not induce CPP, TRPV1 activation is necessary but not sufficient for morphine reward. Furthermore, local microinjection of a selective TRPV1 antagonist into the DSt blocked morphine-CPP, indicating that DSt TRPV1 significantly contributed to morphine reward. The DSt, a component of the nigrostriatal dopamine pathway, was reported to be involved in reward-based learning (Balleine *et al*, 2007; Schmitzer-Torbert and Redish, 2004) and drug seeking (Vanderschuren *et al*, 2005). Electrolytic lesions of either the DStor ventral striatum significantly reduced morphine self-administration, suggesting that these regions have critical roles in the rewarding properties of morphine (Suto *et al*, 2011). As our study only focused on TRPV1 in the DSt, we could not rule out the role of TRPV1 in other brain regions such as the cortex, nucleus accumbens, and ventral tegmental area, which may also participate in morphine reward. These hypotheses will be explored in future studies.

Although the *in vivo* mechanisms of morphine-modulated TRPV1 function remain unclear, studies have demonstrated that morphine modulates TRPV1 function *in vitro* through a cAMP-dependent protein kinase A (PKA) pathway (Vetter *et al*, 2006). Chronic morphine treatment

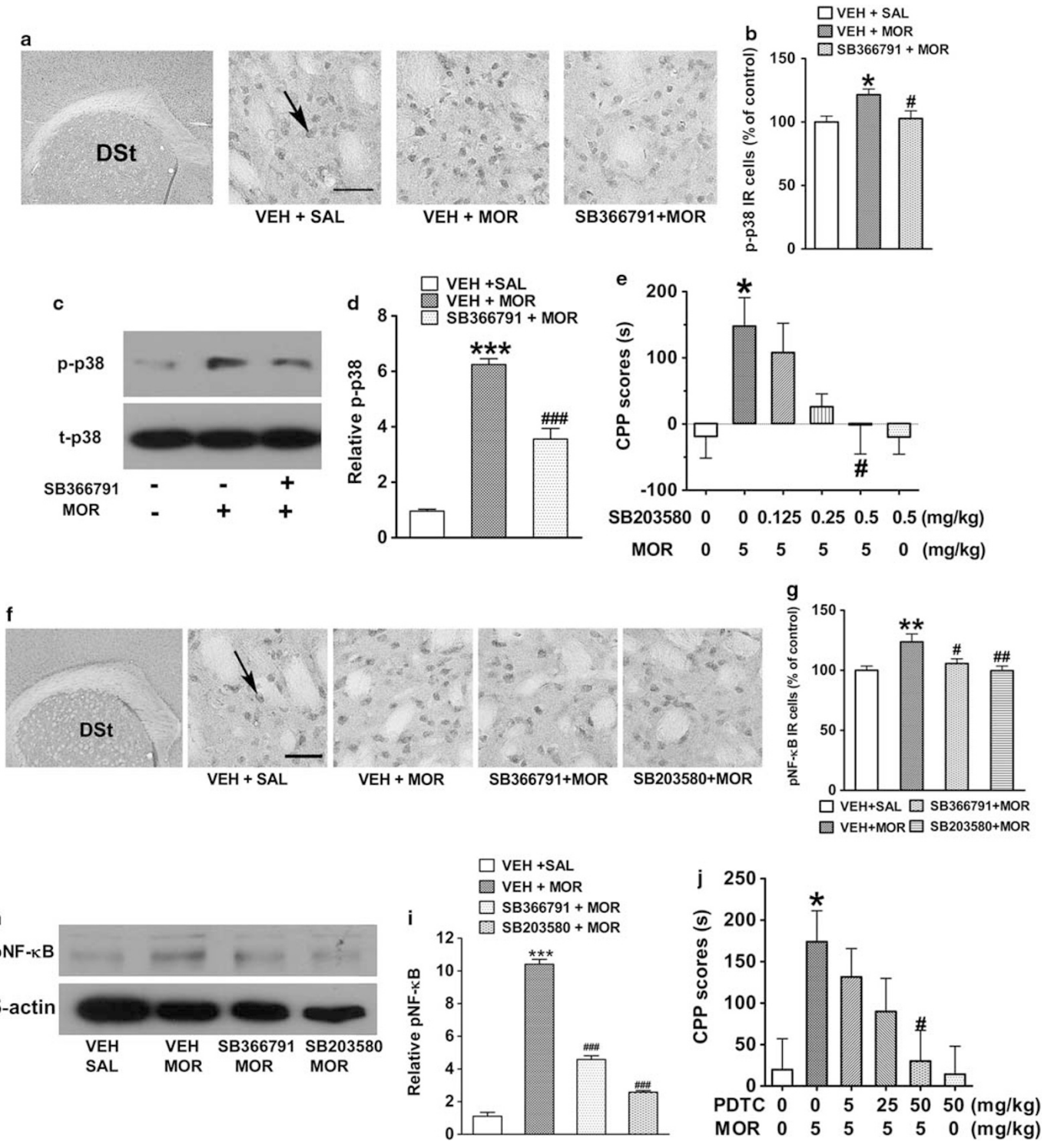

Figure 5 Effects of a transient receptor potential vanilloid type 1 (TRPV1) antagonist on p38/nuclear factor kappa B (NF-κB) in the dorsal striatum (DSt) of morphine-conditioned place preference (CPP) mice. (a, b) Photomicrographs and quantitative analysis of the number of p-p38 IR cells ($n = 6$/group). (c, d) Representative images and quantitative analysis of p-p38 levels in the DSt ($n = 3$/group). (e) Effects of a selective p-p38 inhibitor on morphine-CPP in mice ($n = 12$–13/group). (f, g) Photomicrographs and quantitative analysis of the number of pNF-κB IR cells ($n = 6$/group). (h, i) Representative images and quantitative analysis of pNF-κB levels in the DSt ($n = 3$/group). (j) Effects of a selective pNF-κB antagonist on morphine-CPP in mice ($n = 9$–10/group). Arrows indicate a p-p38 IR cell (a) and a pNF-κB cell (f); scale bars, 40 μm. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *vs* VEH + SAL; $^{\#\#\#}P < 0.001$; $^{\#\#}P < 0.01$; $^{\#}P < 0.05$ *vs* VEH + MOR. MOR, morphine; SAL, saline; VEH, vehicle.

increased the activity of cAMP-dependent protein kinases such as PKA and PKC (Terwilliger *et al*, 1991). Activity of the TRPV1 channel was found to be modulated by PKA and PKC (Tominaga *et al*, 1998); thus, morphine might modulate *in vivo* TRPV1 function through PKA, PKC, or both pathways.

There are at least three possible mechanisms underlying the effects of TRPV1 antagonists on morphine reward. One possible mechanism is that TRPV1 affects μ-opioid receptor binding, which is increased by morphine. Fattore *et al* (2007) demonstrated that [^{3}H]DAMGO binding in the CPu was remarkably elevated in rats self-administering heroin compared with controls. Similarly, our data also indicated that [^{3}H]DAMGO binding in the DSt increased significantly in mice with a preference for the morphine-paired compartment. The increased binding of μ-opioid receptors was diminished by a TRPV1 antagonist.

The second possible mechanism is that a TRPV1 antagonist suppresses morphine-induced AC1 upregulation, thereby preventing the upregulation of the cAMP signaling. Upregulation of the cAMP pathway in the NAc reportedly contributes to morphine reward (Kelley and Holahan, 1997), and repeated morphine administration upregulates AC1 and AC8 (Lane-Ladd *et al*, 1997). Knocking out both the *AC1* and *AC8* genes significantly reduces morphine-CPP, suggesting that AC1, AC8, or both are necessary for morphine-CPP (Li *et al*, 2006b). Our data show that upregulation of AC1 but not AC8 in the DSt is involved in morphine-CPP. We could not rule out the involvement of other AC isoforms in morphine reward. AC1, a Ca^{2+}-activated enzyme, is stimulated by low Ca^{2+} concentrations (150–200 nM), and is approximately five times more sensitive to Ca^{2+} than AC8 (Nielsen *et al*, 1996). The TRPV1 channel is highly permeable to Ca^{2+}, which is similar to the NMDA receptor channel, and thus TRPV1 can trigger Ca^{2+}-mediated cell signaling (Kauer and Gibson, 2009). Simultaneous upregulation of the highly Ca^{2+}-permeable TRPV1 channels and Ca^{2+}-stimulated AC1 in the DSt may be involved in morphine reward. Therefore, TRPV1 antagonists might inhibit morphine reward in two distinct ways such as (1) by blocking TRPV1 to reduce intracellular Ca^{2+} levels, which activates AC1, and (2) by suppressing morphine-induced AC1 upregulation.

A third possible mechanism may be the blockade of morphine reward by the TRPV1 antagonist via the inhibition of p38/NF-κB signaling. The p38/NF-κB signaling pathway in the nucleus accumbens has a critical role in morphine reward in rats (Zhang *et al*, 2011). Similarly, our data showed that repeated morphine administration increases p38 and NF-κB phosphorylation in the DSt of morphine-CPP mice. In addition, we found that a p38 inhibitor repressed morphine-induced increases in NF-κB phosphorylation, implicating p38 as an upstream regulator of NF-κB in morphine reward. NF-κB is a nuclear transcription factor and is reportedly regulated by p38 (Song *et al*, 2006). Phosphorylation of NF-κB can enhance its DNA-binding ability and consequently alter target gene expression (Song *et al*, 2006), which may underlie the long-term neuroadaptive changes induced by morphine. Besides its role in immune and inflammatory responses, brain NF-κB was recently implicated in synaptic plasticity, learning, and memory (Meffert and Baltimore, 2005). Activation of p38 and NF-κB is required for reward-related learning and memory of drugs; in rats, inhibition of p38 blocked reward-related learning of methamphetamines (Gerdjikov *et al*, 2004), whereas a NF-κB inhibitor prevented reward-related memory of morphine (Yang *et al*, 2011). In present study, we found that a TRPV1 antagonist suppressed morphine-induced increases in phosphorylation of p38 and NF-κB in the DSt. Taken together, our findings suggest that blockade of p38/NF-κB signaling in the DSt may underlie the effects of TRPV1 antagonists on morphine-CPP.

In conclusion, our data demonstrate that morphine-induced TRPV1 upregulation in the DSt significantly contributes to morphine reward. We also found that increased μ-opioid receptor binding, AC1 upregulation, and p38/NF-κB activation accompany morphine reward via TRPV1. The diagram in Supplementary Figure S1 illustrates the possible mechanism by which TRPV1 modulates morphine reward. TRPV1 might be a promising therapeutic target for treating morphine dependence.

FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)