



## ARTICLE

# Upregulation of dynorphin/kappa opioid receptor system in the dorsal hippocampus contributes to morphine withdrawal-induced place aversion

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Aversive emotion of opioid withdrawal generates motivational state leading to compulsive drug seeking and taking. Kappa opioid receptor (KOR) and its endogenous ligand dynorphin have been shown to participate in the regulation of aversive emotion. In the present study, we investigated the role of dynorphin/KOR system in the aversive emotion following opioid withdrawal in acute morphine-dependent mice. We found that blockade of KORs before pairing by intracerebroventricular injection of KOR antagonist norBNI (20, 40  $\mu$ g) attenuated the development of morphine withdrawal-induced conditioned place aversion (CPA) behavior. We further found that morphine withdrawal increased dynorphin A expression in the dorsal hippocampus, but not in the amygdala, prefrontal cortex, nucleus accumbens, and thalamus. Microinjection of norBNI (20  $\mu$ g) into the dorsal hippocampus significantly decreased morphine withdrawal-induced CPA behavior. We further found that p38 MAPK was significantly activated in the dorsal hippocampus after morphine withdrawal, and the activation of p38 MAPK was blocked by pretreatment with norBNI. Accordingly, microinjection of p38 MAPK inhibitor SB203580 (5  $\mu$ g) into the dorsal hippocampus significantly decreased morphine withdrawal-produced CPA behavior. This study demonstrates that upregulation of dynorphin/KOR system in the dorsal hippocampus plays a critical role in the formation of aversive emotion associated with morphine withdrawal, suggesting that KOR antagonists may have therapeutic value for the treatment of opioid withdrawal-induced mood-related disorders.

**Keywords:** morphine withdrawal; conditioned place aversion; kappa opioid receptor; p38 MAPK; norBNI; dorsal hippocampus

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## INTRODUCTION

Opioid addiction is a chronic relapsing disorder characterized by compulsive drug taking, persistent drug craving and loss of control over drug craving [1]. Drug withdrawal-induced aversive emotion is a critical factor for induction of motivational state [2–4]. The mechanism for generation of such aversive emotion associated with drug withdrawal remains unclear. We previously demonstrated that the dorsal hippocampus was required for the acquisition and consolidation of the aversive memory of morphine withdrawal [5]. Excitotoxic damage to the dorsal hippocampus impaired morphine withdrawal-induced place aversion behavior [5]. This finding supports that the dorsal hippocampus is crucial for the formation of aversive emotion, but its exact role is unknown yet.

Kappa opioid receptors (KORs) are widely expressed in the central nervous system [6–8], and they play a key role in the regulation of different pharmacological effects such as analgesia, antipruritic, blocking psychostimulant effects and aversive emotion including anxiety, depression and dysphoria [9–14]. Activation

of KORs produced conditioned place aversion behavior [15–18] and caused an increase in the electrical stimulation thresholds in mice [19, 20]. In recent years, the role of KORs and its endogenous ligand dynorphin in mood-related disorders associated with drug withdrawal has received wide attention. It was reported that ethanol withdrawal-induced anxiety, cocaine withdrawal-induced depressive-like behaviors and heroin abstinence-induced emotional disruption were all in part regulated by dynorphin/KOR system [21–25]. We recently found that antagonism of KORs reversed depressive-like behaviors induced by morphine withdrawal [26, 27]. These studies clearly support that dynorphin/KOR system is involved in drug withdrawal-induced mood-related disorders, but it is still unclear how dynorphin/KOR system modulates emotional disorders after drug withdrawal. Moreover, most of the existing studies focus on chronically dependent animals. Whether, in acutely dependent animals, dynorphin/KOR system is also involved in morphine withdrawal-induced aversive emotion remains unclear.

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Conditioned place aversion (CPA), a model of Pavlovian associative learning, is a sensitive measurement for the negative motivational state produced by acute opioid dependence and withdrawal [5, 28–30]. In the present study, CPA was applied to explore the neurobiological mechanisms underlying the development of aversive emotion induced by conditioned morphine withdrawal (CMW) and the potential role of dynorphin/KOR system.

## MATERIALS AND METHODS

### Animals

Male C57BL/6 mice (20–25 g) were purchased from the Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). The mice were housed five per cage and maintained on a 12 h light/dark cycle with access to food and water. All experimental procedures were approved by the Animal Care and Use Committee of Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

### Drugs and antibodies

Morphine Hydrochloride was obtained from Shenyang First Pharmaceutical Co. Ltd (Shenyang, Liaoning, China). NorBNI was purchased from Abcam. Naloxone hydrochloride and SB203580 were purchased from Sigma Aldrich. The dynorphin A antibody was obtained from Abcam and was diluted to 1:1000. Anti-p38 and anti-p-p38 antibodies were supplied by Cell Signaling Technology and were diluted to 1:1000 for the Western blot analyses. HRP-goat anti-rabbit IgG antibody and HRP-goat anti-mouse IgG antibody were purchased from Santa Cruz Biotechnology. The actin-specific antibody was acquired from Sigma Aldrich and was diluted to 1:10000 for the Western blot.

### Conditioned place aversion

The conditioned place aversion (CPA) apparatus [32 cm (length) × 16 cm (width) × 38 cm (height)] was divided into two equal-sized compartments separated by a removable board (6 cm × 6 cm) that allowed the mice to have free access to each compartment. The two compartments were distinguished by visual and tactile cues. One compartment has a black wall and smooth floor, whereas the other compartment has a white wall and textured floor. A camera was placed above the middle of the apparatus to record animal activity.

The CPA paradigm consists three phases: preconditioning, conditioning and testing. In the preconditioning phase, mice were allowed to freely explore the entire apparatus for 15 min, and the time spent in each compartment was recorded. The measurement and data were analyzed with DigBehav Animal Behavioral Analysis System (Shanghai Jiliang Software Technology, Shanghai, China). If the time that the mice spent in either compartment was >480 s, this compartment was defined as the drug-paired compartment. The mice those exhibited strong unconditioned aversion (<180 s) toward either compartment were eliminated from the study. The conditioning phase occurred over the next 2 days. Mice were injected with saline (10 mL/kg, sc) and assigned to the unfavored compartment for 30 min. Then, the mice were injected with either morphine (20 mg/kg, sc) or saline (10 mL/kg, sc), and four hours later they were injected with either naloxone (0.2 mg/kg, sc) or saline and then confined to the preferred compartment for 30 min. This compartment would be referred as the “drug treatment-paired compartment”. In the testing phase (24 h after the last conditioning trial), each mouse was allowed to freely explore the entire apparatus for 15 min, and the time spent in each compartment was recorded. The CPA score (aversion score) was calculated as the time spent in the drug-paired compartment during the testing phase minus the time spent in the drug-paired compartment during the preconditioning phase.

### Novel object recognition test

Novel object recognition (NOR) test consisted of three phases: habituation (day 1–3), training (day 4), and testing (day 5). In the habituation phase, animals were allowed to freely explore the apparatus [20 cm (length) × 20 cm (width) × 40 cm (height)] for 10 min. In the training phase, the animals are exposed to the familiar arena with two identical objects (FL, FR) placed at an equal distance for 10 min. In the testing phase (24 h after training phase), the mice are allowed to explore the apparatus in the presence of the familiar object (F) and a novel object (N) for 10 min. During the test phase, the time spent exploring the familiar object (TF) and novel object (TN) was recorded. Object exploration was defined when the mice place their nose towards the object at a distance of less than 2 cm. The discrimination index (DI) was calculated according to the formula:  $DI = (TN - TF)/(TN + TF)$ .

### Cannulation and microinjections

Mice were anesthetized with sodium pentobarbital (80 mg/kg, ip) and placed in a stereotaxic apparatus (Ruiwode, Shenzhen, Shanghai) with nonpuncture ear bars. Mice were implanted bilaterally with guide cannula (26 gauge) in the dorsal hippocampus [anteroposterior (AP), –2.05 mm; mediolateral (ML), ±1.6 mm; dorsoventral (DV), –1.3 mm]. The cannulae were anchored to the skulls with three stainless-steel screws and dental cement. A stainless-steel blocker was inserted into each cannula to ensure patency and prevent infection. Mice were allowed 7 days for recovery in their home cages before any experiments. Mice were bilaterally injected in the guide cannula by a 31-gauge dummy cannula (Plastics One, USA) that extended 1.5 mm longer than the tip of guide cannula, which was connected to a 10 µL microsyringe mounted in the microinfusion pump (Harvard Apparatus, USA). The microinfusion rate was 200 nL/min. Internal cannulae were removed at least 2 min after injection for drug diffusion. NorBNI was dissolved in PBS with 5% DMSO to a final concentration of 20 µg/µL or 10 µg/µL. SB203580 was dissolved in DMSO (50 µg/µL stock solution) and diluted in PBS to a final concentration of 5 µg/µL before using.

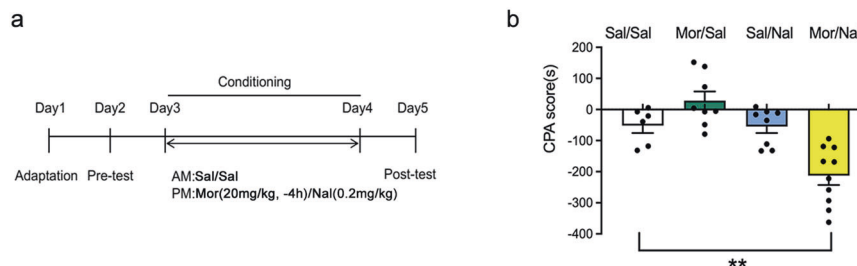
### Histology

After behavior testing, the mice were deeply anesthetized with sodium pentobarbital (120 mg/kg), and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in PBS. The brains were removed and stored in a 30% sucrose/PBS solution for 3 days. Coronal sections (30 µm thick) were cut on a cryostat (Leica, Germany) and stained with cresyl violet. The brain slices were examined by light microscopy to identify the injection sites. Animals were only kept for analysis when the cannulas were placed in the right sites.

### Immunoblotting

The mice were anesthetized with pentobarbital sodium and sacrificed by decapitation. Coronal brain sections (0.5 mm thick) were obtained using a mouse brain slicer (Braintree Scientific Inc, Braintree, MA, USA). Both sides of each dorsal hippocampus, amygdala, nucleus accumbens, prefrontal cortex and thalamus were punched from brain slices using a blunt-end, 16-gauge syringe needle (1-mm inner diameter). The cytoplasm protein fraction was isolated as follows: briefly, the tissue was homogenized with a Teflon pestle in a glass homogenate tube with 150 µL of protein lysis buffer, which contained 1× phosphatase and protease inhibitor. Homogenate was then centrifuged at 1000 × g for 10 min at 4 °C. The supernatant was mixed with 4× loading buffer, then boiled at 95 °C for 10 min. The protein concentration was quantitated by BCA assay.

Equal amounts of protein were subjected to electrophoresis in 12% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with



**Fig. 1** Conditioned place aversion was induced by naloxone-precipitated morphine withdrawal in mice. **a** Timeline for the experimental procedure. **b** Conditioned place aversion was elicited by two pairing with 0.2 mg/kg naloxone in mice exposed to 20 mg/kg morphine. Data are expressed as mean  $\pm$  SEM,  $**P < 0.01$  compared with the saline-treated control group, one-way ANOVA with Bonferroni's *post hoc* test. Sal Saline, Mor Morphine, Nal Naloxone.

5% non-fat milk dilution in TBST for 1 h at room temperature and incubated with primary antibodies against dynorphin (1:1000), p-p38 (1:1000), or p38 (1:1000) dilutions overnight. The membranes were then incubated with HRP-conjugated goat anti-rabbit IgG (1:30000) for 1 h, bands were developed with a chemiluminescent substrate (GE Healthcare). The density of images was quantified using the Image J software. Data were normalized to control group values for all groups.

#### Spontaneous activity

Mice were placed in the spontaneous activity box [20 cm (length)  $\times$  20 cm (width)  $\times$  40 cm (height)] equipped with infrared video recorders for 30 min. The moving distance was analyzed using DigBehav Animal Behavioral Analysis System.

#### Statistical analysis

All data were performed using GraphPad Prism 6. Data are presented as mean  $\pm$  SEM. The data were analyzed with either two-tailed Student's *t*-tests, a one-way ANOVA or a two-way ANOVA followed by Bonferroni *post hoc* tests when appropriate. Differences of  $P < 0.05$  were considered statistically significant.

## RESULTS

### Conditioned morphine withdrawal (CMW) induced significant place aversion behavior

Conditioned place aversion (CPA) can be elicited by pairing with naloxone after morphine [28]. According to our previous protocol [5, 29, 30], we successfully generated CPA by two pairing with naloxone (0.2 mg/kg, sc) 4 h after exposure to morphine (20 mg/kg, sc) (Fig. 1a). It showed that CMW induced significant place aversion behavior, evidenced by that mice spent less time in the withdrawal-associated compartment (CPA score: Sal/Sal,  $-51.72 \pm 23.92$  s,  $n = 6$ ; Mor/Sal,  $28.31 \pm 29.82$  s,  $n = 8$ ; Sal/Nal,  $-54.56 \pm 21.29$  s,  $n = 8$ ; Mor/Nal,  $-213.0 \pm 29.59$  s,  $n = 10$ ;  $F_{(3,28)} = 15.30$ ,  $P < 0.0001$ ; Fig. 1b). In contrast, mice pretreated with morphine, followed by pairing with saline, did not produce CPA. Mice pretreated with saline, followed by pairing with naloxone, also did not produce CPA.

### $\kappa$ opioid receptor antagonist norBNI significantly inhibited morphine withdrawal-induced CPA behavior

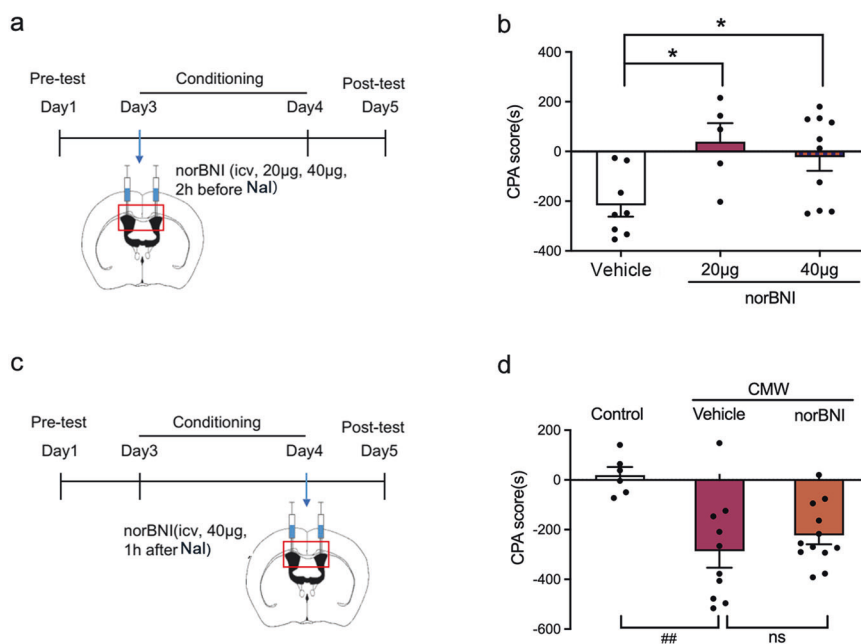
$\kappa$  opioid receptors (KORs) are widely expressed in the brain and play a key role in regulation of aversive emotions. To identify whether KORs were involved in the aversive emotion elicited by morphine withdrawal, we examined the effect of KOR antagonist norBNI on morphine withdrawal-induced CPA. As shown in Fig. 2a, b, norBNI was injected into the lateral ventricle 2 h before naloxone pairing, and we found that both 20  $\mu$ g and 40  $\mu$ g norBNI significantly blocked morphine withdrawal-induced CPA behavior (CPA score: Vehicle,  $-216.5 \pm 45.48$  s,  $n = 8$ ; 20  $\mu$ g of norBNI,

$39.36 \pm 74.32$  s,  $n = 5$ ; 40  $\mu$ g of norBNI,  $-23.36 \pm 54.87$  s,  $n = 10$ ;  $F_{(2,20)} = 5.068$ ,  $P = 0.0166$ ; Fig. 2b). However, we found that norBNI (40  $\mu$ g) injected 1 h after naloxone pairing did not affect mice CPA behavior (*d*: CPA score: Control,  $19.63 \pm 32.07$  s,  $n = 6$ ; Vehicle,  $-287.0 \pm 66.46$  s,  $n = 10$ ; norBNI,  $-223.2 \pm 35.68$  s,  $n = 12$ ;  $F_{(2,25)} = 7.765$ ,  $p = 0.0024$ ; Fig. 2c, d). These data indicate that involvement of KORs in the regulation of CPA formation but not CPA expression associated with morphine withdrawal.

### Upregulation of dynorphin/ $\kappa$ opioid receptor system in the dorsal hippocampus contributed to morphine withdrawal-induced CPA behavior

Considering the importance of KORs in morphine withdrawal-induced CPA behavior, we next determined the key regions whereby KORs modulated CPA induced by morphine withdrawal. We detected the expression level of dynorphin A, the endogenous ligand of KORs, in mice subjected to CPA training in different brain regions related to addiction and emotion. We dissected the dorsal hippocampus, amygdala, nucleus accumbens, prefrontal cortex and thalamus. As shown in Fig. 3, morphine withdrawal significantly increased dynorphin A level in the dorsal hippocampus (Control, 100.0%  $\pm$  15.80%; CMW, 159.3%  $\pm$  20.30%;  $n = 7-11$ ;  $t_{(16)} = 2.318$ ,  $P = 0.0340$ ; Fig. 3a), but not in the amygdala, prefrontal cortex, nucleus accumbens and thalamus (*b*: Control, 100.0%  $\pm$  13.12%; CMW, 106.6%  $\pm$  20.48%;  $n = 6-12$ ;  $t_{(16)} = 0.2147$ ,  $P = 0.8328$ ; *c*: Control, 100.0%  $\pm$  7.589%; CMW, 110.6%  $\pm$  12.65%;  $n = 12$ ;  $t_{(22)} = 0.7206$ ,  $P = 0.4787$ ; *d*: Control, 100.0%  $\pm$  6.077%; CMW, 108.6%  $\pm$  9.679%;  $n = 12$ ;  $t_{(22)} = 0.7558$ ,  $P = 0.4578$ ; *e*: Control, 100.0%  $\pm$  12.14%; CMW, 93.69%  $\pm$  6.314%;  $n = 8-12$ ;  $t_{(18)} = 0.5041$ ,  $P = 0.6203$ ; Fig. 3b-e). To verify the role of the dorsal hippocampus, we microinjected 20  $\mu$ g norBNI into the dorsal hippocampus 2 h before naloxone pairing (Fig. 4a). It was found that norBNI microinjection into the dorsal hippocampus significantly blocked CPA induced by morphine withdrawal, but did not affect mice locomotor activity (*b*: CPA score: Control,  $-25.84 \pm 21.81$  s,  $n = 10$ ; Vehicle,  $-275.9 \pm 18.01$  s,  $n = 10$ ; norBNI,  $-131.1 \pm 54.09$  s,  $n = 8$ ;  $F_{(2,25)} = 16.69$ ,  $P < 0.0001$ ; *c*: distance traveled: Vehicle, 63493  $\pm$  7715 mm; norBNI, 59594  $\pm$  6929 mm,  $n = 8$ ;  $t_{(14)} = 0.3761$ ,  $P = 0.7125$ ; Fig. 4b-e).

To avoid the possibility that the effects of norBNI was due to its ability in modulating mice standard memory tasks, we conducted the novel object recognition test (Fig. 6a). As shown in Fig. 6b, all groups of mice showed similar preference for each object in the training phase (Vehicle-FL: 31.30  $\pm$  7.270 s, FR: 25.39  $\pm$  8.397 s,  $n = 7$ ,  $P > 0.9999$ ; norBNI-FL: 26.72  $\pm$  4.129 s, FR: 28.34  $\pm$  4.545 s,  $n = 6$ ,  $P > 0.9999$ ). Animals were microinjected with vehicle or norBNI (20  $\mu$ g) into the dorsal hippocampus. After 2 h, animals were exposed to a novel object recognition test. Both vehicle- and norBNI-treated mice showed significant preference for the novel object (Fig. 6c, Vehicle-F: 33.60  $\pm$  4.760 s, N: 66.91  $\pm$  10.52 s,  $n = 7$ ,  $P = 0.0276$ ; norBNI-F: 25.32  $\pm$  4.445 s, N: 35.45  $\pm$  4.841 s,



**Fig. 2** Effects of norBNI by intracerebroventricular (icv) administration on conditioned morphine withdrawal-induced CPA behavior. Left column. Timeline for norBNI icv injection before **a** and after **c** naloxone injection and pairing and behavioral testing. Right column. **b** Injection of norBNI before pairing attenuated CPA induced by morphine withdrawal. NorBNI (10 µg/µL, 20 µg/µL, 1 µL/side) or vehicle (1 µL/side) was bilaterally microinjected into icv 2 h before naloxone injection. **d** Injection of norBNI after pairing did not affect CPA induced by morphine withdrawal. NorBNI (20 µg/µL, 1 µL/side) or vehicle (1 µL/side) was bilaterally microinjected into icv 1 h after naloxone injection. Values are expressed as mean ± SEM, \**P* < 0.05 compared with the corresponding vehicle-treated CMW group, ##*P* < 0.01 compared with saline-treated control group, one-way ANOVA with Bonferroni's *post hoc* test. CMW conditioned morphine withdrawal.

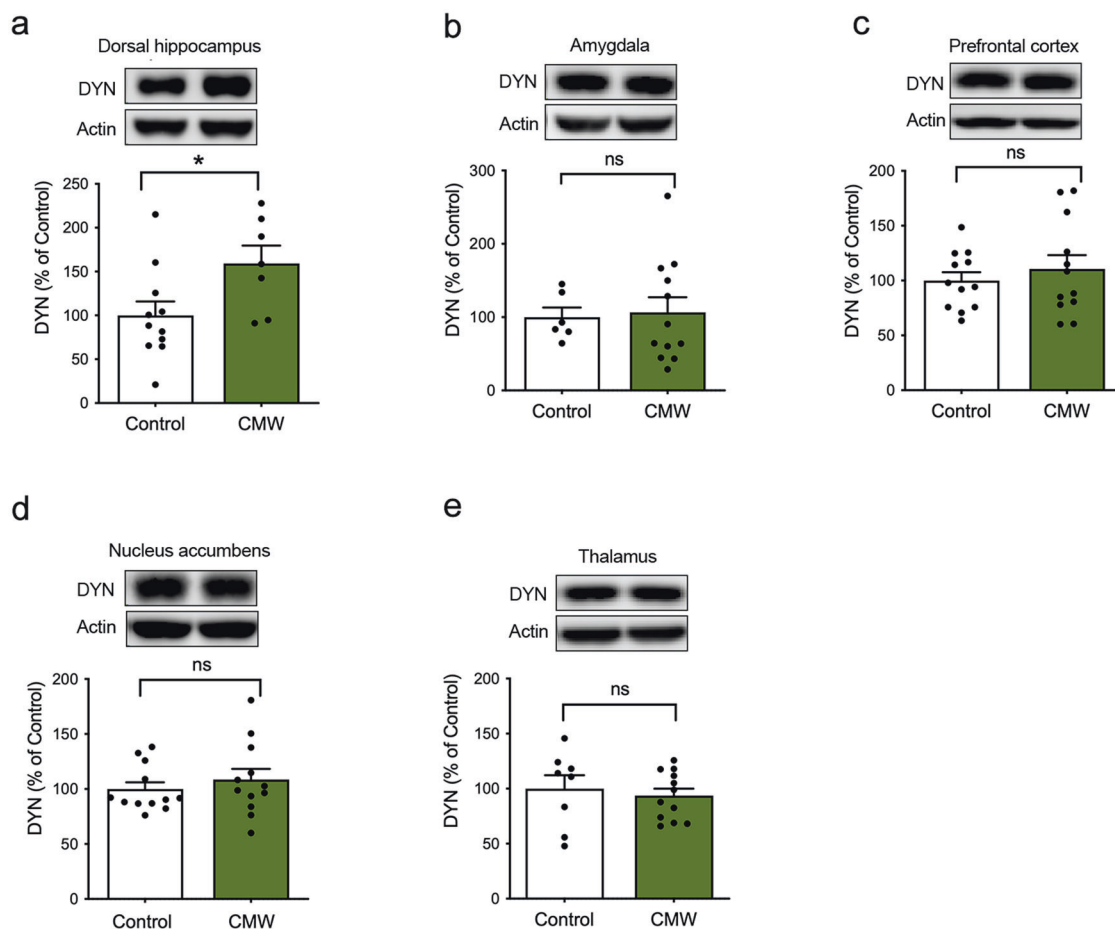
*n* = 6, *P* = 0.0276). There were no significant differences in the discrimination index between groups (Fig. 6d, Vehicle: 0.3264 ± 0.06320 s, norBNI: 0.1691 ± 0.08594 s, *n* = 6–7; *t*<sub>(11)</sub> = 1.503, *P* = 0.1611). The results indicated that norBNI did not affect mice memory and cognitive function.

KOR-mediated p38 MAPK activation was critically involved in morphine withdrawal-induced CPA behavior p38 mitogen-activated protein kinase (p38 MAPK) belongs to the MAPK family and plays an important role in aversive effects of stress [15]. It has been reported that KOR agonist-induced aversion is associated with activation of p38 MAPK in the dorsal raphe nucleus and midbrain [16, 17]. To investigate whether p38 MAPK activation mediated morphine withdrawal-induced CPA behavior, we detected p38 MAPK activation in the dorsal hippocampus after morphine withdrawal. It was found that p38 MAPK was significantly activated in the dorsal hippocampus (Control, 100.0% ± 14.13%; CMW, 154.3% ± 16.93%; *n* = 11; *t*<sub>(20)</sub> = 2.462, *P* = 0.0230; Fig. 5a, left), but not in the amygdala (Control, 100.0% ± 9.597%; CMW, 117.2% ± 10.22%; *n* = 12; *t*<sub>(22)</sub> = 1.227, *P* = 0.2326; Fig. 5a, right), which was consistent with the changes of dynorphin A. We also found that norBNI pretreatment significantly inhibited the activation of p38 MAPK in mice subjected to morphine withdrawal (Control, 100.0% ± 2.619%; Vehicle, 153.5% ± 26.40%; norBNI, 87.55% ± 14.06%; *n* = 5–7; *F*<sub>(2,15)</sub> = 4.901, *P* = 0.0230; Fig. 5b). To verify the role of p38 MAPK activation in morphine withdrawal-induced CPA, p38 MAPK inhibitor SB203580 was used to inhibit the function of p38 MAPK. The experimental design is shown in Fig. 5c, one hour before naloxone pairing, SB203580 (5 µg) were microinjected into the dorsal hippocampus. As shown in Fig. 5d–h, SB203580 pretreatment significantly blocked p38 MAPK activation in the dorsal hippocampus (Control, 100.0% ± 7.284%; Vehicle, 192.7% ± 30.14%; SB203580,

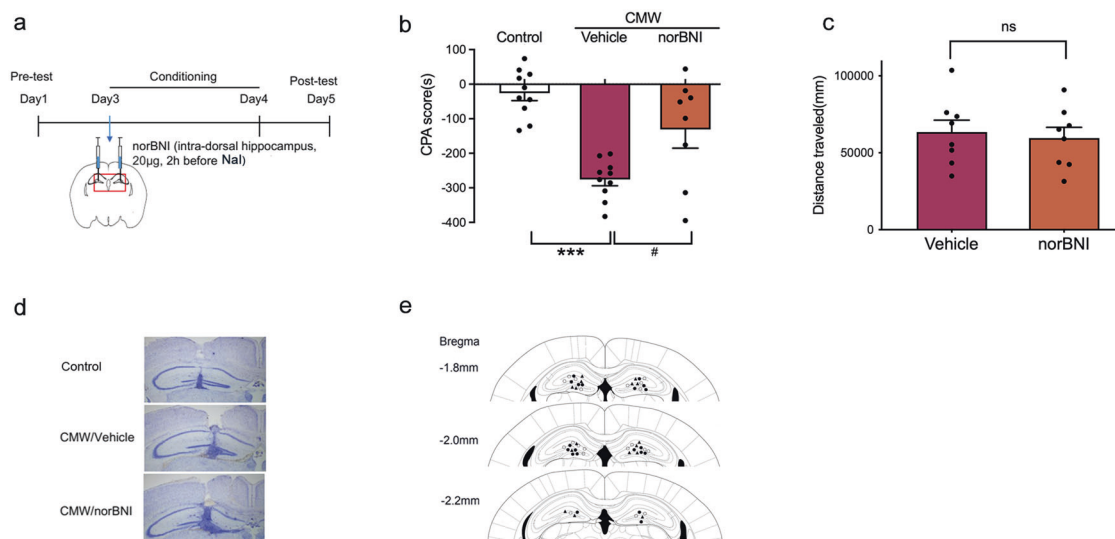
91.11% ± 24.07%; *n* = 6–7; *F*<sub>(2,16)</sub> = 5.877, *P* = 0.0122; Fig. 5d) and consequently inhibited CPA (CPA score: Control, 9.44 ± 27.81 s, *n* = 8; Vehicle, −300.5 ± 25.25 s, *n* = 8; SB203580, −139.7 ± 38.46 s, *n* = 10; *F*<sub>(2,23)</sub> = 20.85, *P* < 0.0001; Fig. 5e), but did not affect mice locomotor activity (distance traveled: Vehicle, 47493 ± 7767 mm; SB203580, 59064 ± 5381 mm, *n* = 8; *t*<sub>(14)</sub> = 1.225, *P* = 0.2409; Fig. 5f). These data indicate that p38 MAPK is an important downstream effector of KOR activation that mediates morphine withdrawal-induced CPA behavior. We also determined the effects of SB203580 on learning and memory in mice. Animals were microinjected with vehicle or SB203580 (5 µg) into the dorsal hippocampus. After 1 h, animals were exposed to a novel object recognition test. We found that SB203580 alone did not modulate mice memory and cognitive function (Fig. 6e, Vehicle-FL: 45.45 ± 3.727 s, FR: 37.52 ± 2.714 s, *n* = 8, *P* > 0.9999; SB203580-FL: 40.67 ± 4.880 s, FR: 42.16 ± 6.278 s, *n* = 7, *P* > 0.9999; Fig. 6f, Vehicle-F: 19.77 ± 3.076 s, N: 32.56 ± 4.202 s, *n* = 8, *P* = 0.0498; SB203580-F: 24.29 ± 4.558 s, N: 35.83 ± 5.612 s, *n* = 7, *P* = 0.0498, Fig. 6g, Vehicle: 0.2456 ± 0.07089 s, SB203580: 0.2081 ± 0.06178 s, *n* = 7–8; *t*<sub>(13)</sub> = 0.3938, *P* = 0.7001).

**DISCUSSION**

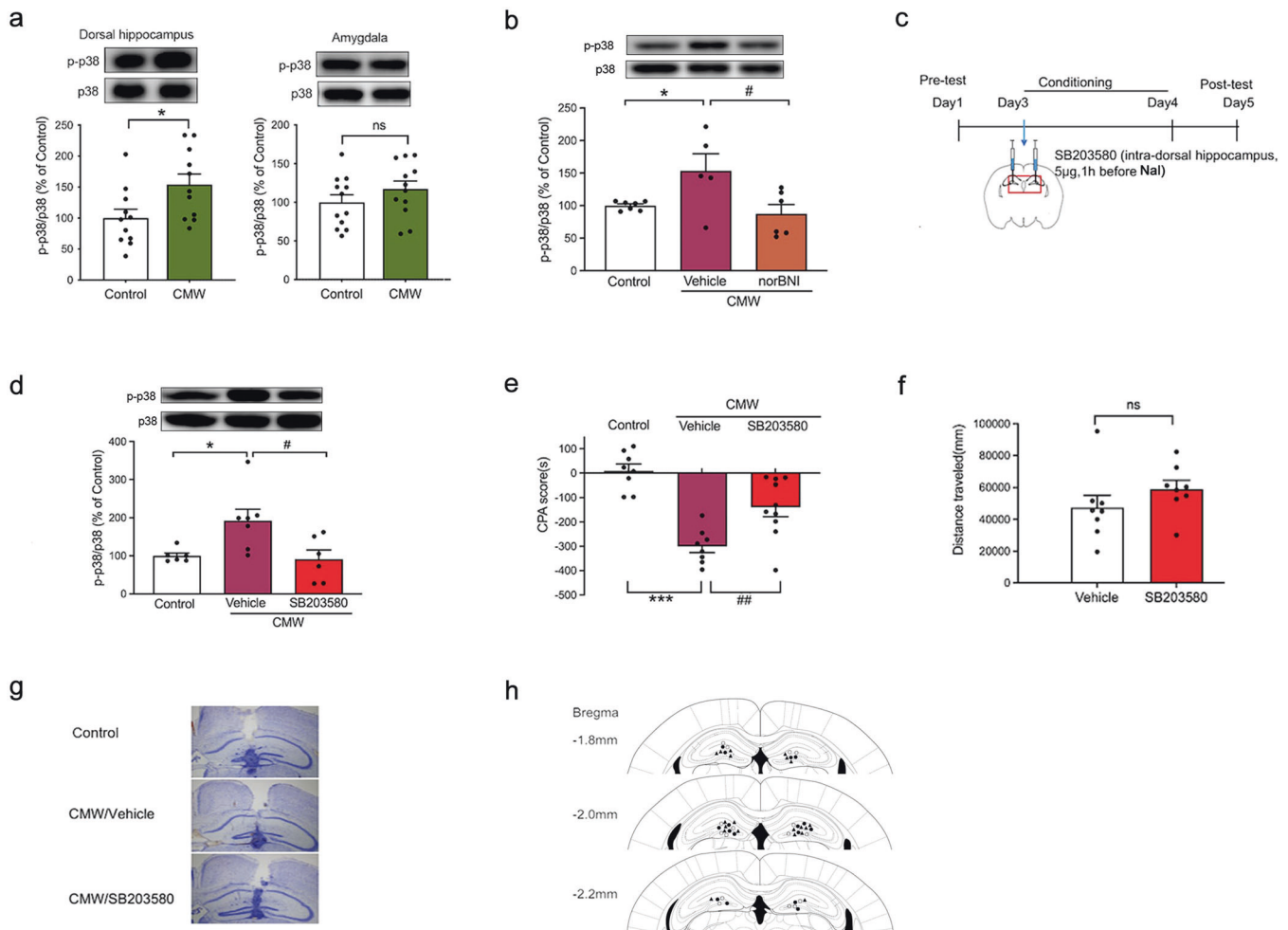
Aversive emotion induced by morphine withdrawal has been shown to play a crucial role in the relapse of addiction. However, the molecular mechanisms that mediate aversive emotion associated with morphine withdrawal are unclear. Here, in the present study, we found that upregulation of dynorphin/KORs in the dorsal hippocampus contributed to morphine withdrawal-induced CPA behavior. p38 MAPK was an important downstream effector of KOR activation. Antagonism of KORs or p38 MAPK in the dorsal hippocampus significantly inhibited morphine withdrawal-induced CPA behavior, without affecting mice memory and cognitive function.



**Fig. 3 The expression levels of dynorphin A in different brain regions after morphine withdrawal.** The dorsal hippocampus (a), amygdala (b), prefrontal cortex (c), nucleus accumbens (d) and thalamus (e) were extracted 1 h after naloxone-precipitated morphine withdrawal. The protein level of dynorphin A was measured by Western blot. Values are expressed as mean  $\pm$  SEM,  $*P < 0.05$  compared with control group in unpaired two-tailed *t*-test. CMW conditioned morphine withdrawal.



**Fig. 4 Effects of norBNI by intra-dorsal hippocampal administration on morphine withdrawal-induced CPA behavior.** a Timeline for the norBNI injection and behavioral testing. b Intra-dorsal hippocampal injection of norBNI before pairing attenuated CPA induced by morphine withdrawal. NorBNI (20  $\mu\text{g}/\mu\text{L}$ , 0.5  $\mu\text{L}/\text{side}$ ) or vehicle (0.5  $\mu\text{L}/\text{side}$ ) was bilaterally microinjected into the dorsal hippocampus 2 h before naloxone injection. c Mice locomotor activity was measured. Nissl staining (d) and schematic representation of injection sites (e  $\circ$ , control;  $\bullet$ , vehicle+CMW;  $\blacktriangle$ , norBNI+CMW) in the dorsal hippocampus for mice used in the experiments. Values are expressed as mean  $\pm$  SEM,  $***P < 0.001$  compared with the saline-treated control group,  $\#P < 0.05$  compared with vehicle-treated CMW group, one-way ANOVA with Bonferroni's *post hoc* test. CMW conditioned morphine withdrawal.



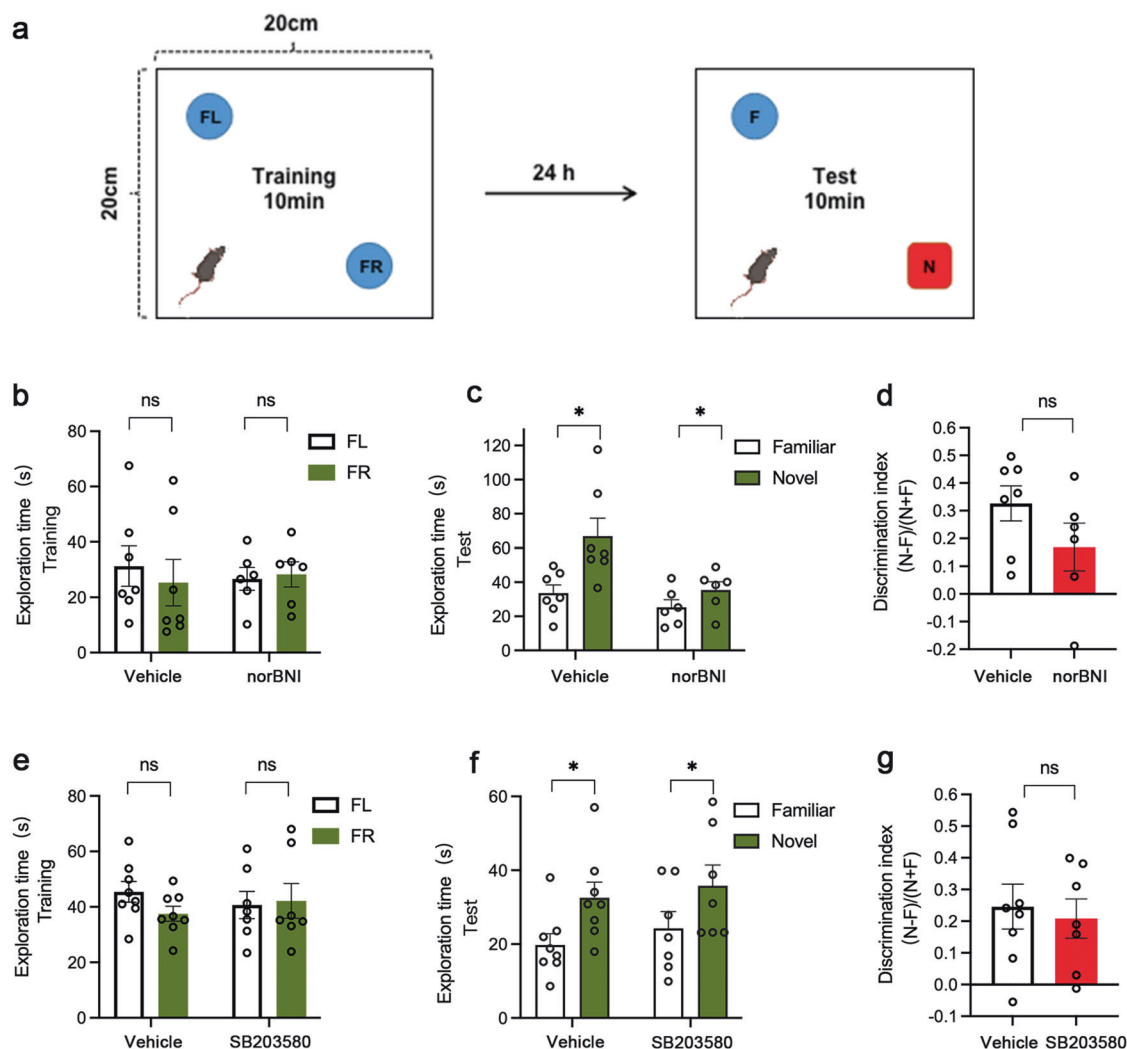
**Fig. 5** KOR dependent p38 MAPK activation was involved in the CPA produced by morphine withdrawal. **a** p38 MAPK was activated in the dorsal hippocampus after morphine withdrawal. **b** Intra-dorsal hippocampal injection with norBNI attenuated p38 MAPK activation. Effects of p38 MAPK inhibitor SB203580 on morphine withdrawal-induced p38 MAPK activation and CPA behavior. **c** Timeline for SB203580 injection and pairing. **d** Intra-dorsal hippocampal injection of SB203580 before pairing attenuated p38 MAPK activation and CPA induced by morphine withdrawal. SB203580 (5  $\mu\text{g}/\mu\text{L}$ , 0.5  $\mu\text{L}/\text{side}$ ) or vehicle (0.5  $\mu\text{L}/\text{side}$ ) was bilaterally microinjected into the dorsal hippocampus 1 h before naloxone injection. **e** Mice locomotor activity was measured. Nissl staining (**g**) and schematic representation of injection sites (**h** ○, control; ●, vehicle + CMW; ▲, SB203580 + CMW) in the dorsal hippocampus for mice used in the experiments. Values are expressed as mean  $\pm$  SEM, \* $P < 0.05$ , \*\*\* $P < 0.001$  compared with saline-treated control group, # $P < 0.05$ , ## $P < 0.01$  compared with vehicle-treated CMW group, one-way ANOVA with Bonferroni's *post hoc* test. CMW conditioned morphine withdrawal.

The hippocampus, particularly the dorsal hippocampus, has been shown to be involved in the modulation of mood-related disorders including anxiety and depression [31, 32]. We previously found that the dorsal hippocampus and amygdala were required for the acquisition and consolidation of aversive emotion induced by morphine withdrawal in rats, since the aversive emotion was impaired by lesions of either the dorsal hippocampus or the amygdala [5]. In this study, we further proved the involvement of the dorsal hippocampus in aversion of morphine withdrawal in mice.

Dynorphin/KORs system controls emotional responses, particularly during stress. Behavioral studies in rodents show that KORs modulate depression and anxiety-related behaviors [23]. Accumulating evidence shows that dynorphin/KORs is also involved in mediating mood related-behaviors associated with ethanol or cocaine withdrawal. We recently further proved that prolonged morphine withdrawal caused depressive-like behaviors via upregulation of dynorphin/KOR signaling in the amygdala [27]. In this study, we found that the expression level

of dynorphin A was significantly increased in the dorsal hippocampus, and antagonism of KORs by intracerebroventricular or intra-dorsal hippocampal injection of norBNI significantly inhibited morphine withdrawal-induced CPA behavior, supporting the role of KORs in regulating reward and mood. Notably, in this study, we found that norBNI inhibited CPA only when it was given before naloxone injection and pairing. When norBNI was given after naloxone injection, it did not produce inhibitory effects. These data suggest that KORs in the dorsal hippocampus are involved in the formation, but not in the expression of CPA to morphine withdrawal. Our study was consistent with previous study showing that KOR antagonism before stress but not after stress blocked negative mood disorders following stress [33].

Moreover, we found that morphine withdrawal-induced p38 MAPK activation was inhibited by KOR antagonist norBNI pretreatment, and microinjection of p38 MAPK inhibitor SB203580 into the dorsal hippocampus significantly reduced the level of p38 MAPK phosphorylation and inhibited morphine



**Fig. 6** Effects of norBNI and SB203580 in the dorsal hippocampus on the novel object recognition test task. **a** Experimental scheme. **b, e** Exploration time during the training phase. **c, f** Exploration time during the test phase. Microinjection of norBNI (20  $\mu\text{g}/\mu\text{L}$ , 0.5  $\mu\text{L}/\text{side}$ ) or vehicle (0.5  $\mu\text{L}/\text{side}$ ) into the dorsal hippocampus 2 h before test. Microinjection of SB203580 (5  $\mu\text{g}/\mu\text{L}$ , 0.5  $\mu\text{L}/\text{side}$ ) or vehicle (0.5  $\mu\text{L}/\text{side}$ ) into the dorsal hippocampus 1 h before test. All groups show more significant preference for the new object than the familiar object. **d, g** Discrimination indexes are plotted as a function of the drugs. There are no differences in discrimination index between vehicle-treated and norBNI-treated or SB203580-treated group. Values are expressed as mean  $\pm$  SEM, \* $P < 0.05$  compared with vehicle-F, norBNI-F, or SB203580-F group, two-way ANOVA with Bonferroni *post hoc* tests. F Familiar, N Novel.

withdrawal-induced place aversion behavior. The data support previous findings that p38 MAPK is a mediator for KOR activation-induced aversion [17, 18, 34–36].

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#### AUTHOR CONTRIBUTIONS

YJW, CXY, and JGL designed the experiments. YC and CYW performed the experiments with the assistance of GYZ, SY, YZD, XLS, WWW, and YM, YC, CYW, and YJW performed data statistical analysis. YC, GYZ, and YJW wrote the manuscript, and YJW, CXY, and JGL revised it.

#### ADDITIONAL INFORMATION

**Competing interests:** The authors declare no competing interests.

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