Research Article

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Effects of Penehyclidine Hydrochloride on Morphine Conditional Place Preference and the Expression of β-Arrestin-2 in Related Brain Regions in Mice

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ABSTRACT

Objective: To determine the effect of penehyclidine hydrochloride (PHC) on the expression, extinction, and reinstatement of morphine-induced conditioned place preference (CPP) and levels of β-arrestin-2 protein expression in different brain regions in mice.

Methods: Six-week-old male Kunming mice were used. The CPP experiment was divided into three stages: establishment; regression; and reproduction. In each stage, the mice were randomly divided into control, morphine-dependent, and PHC groups, with 6 mice per stage. The PHC group received an intraperitoneal injection of PHC 30 min before the test. The control and morphine-dependent groups were treated with vehicle. In the CPP regression stage, the mice were continuously injected with normal saline to extinguish the CPP effect. The PHC group received an intraperitoneal injection of PHC 30 min before the test. The control and morphine-dependent groups were treated with vehicle. Western blot was used to detect β-arrestin-2 protein in the ventral dorsal tegmentum, nucleus accumbens, and prefrontal cortex.

Results: At the CPP establishment stage, the morphine-dependent group had a significantly higher residence time in the gray area than the control group ($P<0.05$). Compared with the morphine-dependent group, the PHC group had a significantly lower residence time in the gray zone ($P<0.05$). The expression of β-arrestin-2 in the ventral dorsal tegmentum and nucleus accumbens was upregulated in the morphine-dependent group ($P<0.01$). The expression of β-arrestin-2 was downregulated in the penehyclidine hydrochloride group ($P<0.01$). At the CPP regression stage, there was no significant difference in grey zone residence time among the three groups. The expression of β-arrestin-2 was upregulated in the ventral dorsal tegmentum and nucleus accumbens in the morphine-dependent group ($P<0.01$). Compared with the control group, the morphine-dependent group had a significantly higher residence time in the gray area during the CPP recurrence stage ($P<0.05$). Compared with the morphine-dependent group, the PHC group had a significantly lower residence time in the gray zone ($P<0.05$). The expression of β-arrestin-2 in the ventral dorsal tegmentum and nucleus accumbens was upregulated in the morphine-dependent group ($P<0.01$, $P<0.05$). The expression of β-arrestin-2 was downregulated in the penehyclidine hydrochloride group ($P<0.01$).

Conclusion: Penehyclidine hydrochloride inhibited morphine-induced CPP and recurrence of morphine-induced CPP. The level of β-arrestin-2 expression was dependent on acute treatment with PHC in the CPP establishment, regression, and recurrence stages, and correlated with the location in different brain regions. This study provides a theoretical basis for promoting the clinical application of PHC.

KEYWORDS: Penehyclidine hydrochloride; Morphine; Psychological addiction; Conditioned place preference; Beta arrestin-2

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INTRODUCTION

Relapse behavior in substance dependence is a special mental effect produced by the central nervous system and is a challenge that must be addressed in the treatment of drug addiction [1,2]. Many studies have confirmed the importance of relapse behavior [2-6]. The central and peripheral cholinergic nervous systems have an important role in the initiation and development of morphine dependence, the agonists of which exacerbate opioid withdrawal symptom [4]. Clinical practice has shown that the muscarinic acetylcholine receptor (mAChR) antagonist, scopolamine, significantly alleviates withdrawal symptoms [7,8]. Penehyclidine hydrochloride (PHC; brand name, Jantonin) is a novel selective anti-M1 and -M3 receptor anticholinergic drug with central sedative effects [5]. Experimental studies on the use of PHC in morphine dependence have not been reported. *β*-arrstin-2 was discovered by purification of *β*-adrenergic receptor kinases [6]. *β*-arrstin-2 regulates the desensitization and internalization of G-protein-coupled receptors, including mAChR, and acts as a protein of the kinase signaling pathway to regulate the immune response [9]. Our group has previously reported that *β*-arrestin-2 is involved in regulating the camp-response element binding protein (CREB)-related signaling pathway and thus affects the biological expression of the CREB-related signaling pathway [7]. In this study on the basis of existing work in *β*-arrestin-2 as the breakthrough point induced by the morphine conditioned place preference (CPP) animal model, we determined the effects of PHC on morphine-induced CPP and its recurrence induced by morphine injection. Furthermore, during morphine-induced CPP, regression, and reappearance of morphine-induced CPP, *β*-arrestin-2 was detected in the brain regions associated with addiction. Expression of *β*-arrestin-2 changes in the ventral tegmental area (VTA), nucleus accumbens (NAc), and prefrontal cortex (PFC) were observed. We conducted a preliminary evaluation of three brain areas for *β*-arrestin-2 expression in different stages of morphine dependence and the mechanism underlying PHC intervention of morphine addiction behavior.

MATERIALS AND METHODS

The Material

Experimental animals

Fifty-four SPF male Kunming mice, weighing 20-25 g, were purchased from the Hubei Provincial Center for Disease Control and Prevention (Hubei, China). The experimental animal license number was SCXK (E) 2012-2014. The temperature and humidity in the animal feeding room were 23±1°C and 55±5%, respectively. The animals had access to food and water ad libitum. The light cycle was 12 hours with the light on at 7:00 AM. The mice were adapted for 1 week, and the experimental personnel touched the animals every other day to reduce the influence of stress on the animals. All experimental procedures were approved by the Animal Ethics Committee of Zhongnan Hospital and Research Centre and the Animal Ethics Committee of Wuhan University, Hubei, China, and performed in accordance with the National Institutes of Health "Guidelines for the Care and Use of Laboratory Animals". The study was carried out in compliance with the ARRIVE guidelines.

Main Reagents and Instruments

Morphine hydrochloride was purchased from Hubei Changao Pharmaceutical Co., LTD (Batch number: 21535-47-7; Hubei, China). Penehyclidine hydrochloride was purchased from Shanghai Yubo Biotechnology Co., LTD. (Batch number: 151937-76-7; Shanghai, China). Anti-*β*-arrestin-2 (ABS137054) and anti-*β*-actin (ABS132001) antibodies were purchased from Erbixin (Shanghai) Biotechnology Co., LTD. (Shanghai, China). The CPP system consists of a sound isolation box, a shuttle box, and a video recording system. The sound insulation box (70 cm long, 70 cm wide, and 140 cm high) has 2 layers of wood lined with soundproof cotton and noise reduction fans on the side. The lower part of the front wall of the speaker box is freely opened to house the shuttle box. Cameras and energy-saving lights are placed on the top of the shuttle box. A box with an inner diameter of 40 cm, a width of 20 cm, and a height of 20 cm in the shuttle box is divided into 2 cubes by a partition board in the middle with a side length of 15 cm, and 2 side is painted black, which is called the black area. The other side is painted gray and is called the gray area. The video recording system consists of a camera (Nanshen High-tech TD-203A; South Shenshi High-tech company, Shenzhen, Guangdong province, China), a lens (Avenir, 3.5-8.0 mm, F1.4; SEIKO ccompany, Shanghai, China) and a computer. A USB video capture card (Syntek STK1160; Taixin Semiconductor company, Shenzhen, Guangdong province, China) was connected to the computer to record the residence time of mice in the black and gray zones.

Methods

The CPP Experiment

CPP experimental Process and Grouping

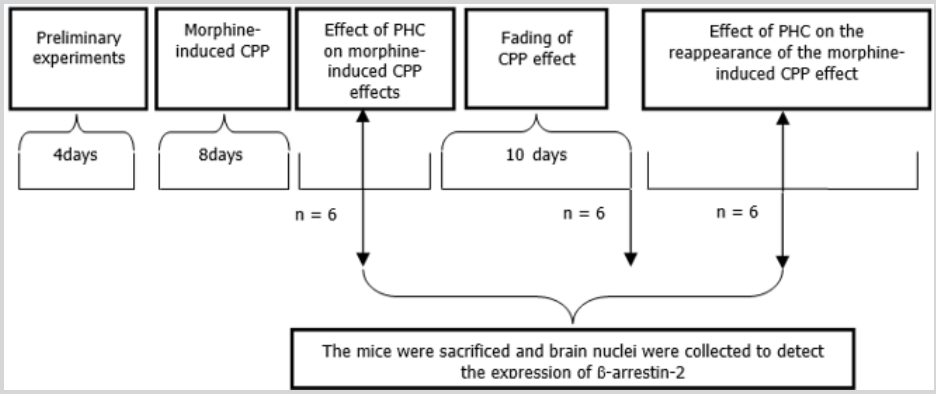

Figure 1: CPP experiment and sampling process.

A biased design was adopted in this experiment. One side of the natural preference of mice in the pre-experiment was used as the non-drug companion area during training, and the other side was used as the drug companion area. The CPP experiment was divided into three stages (Figure 1): ① effect of penehyclidine hydrochloride on morphine-induced CPP effects; ② regression of morphine-induced CPP effects; and ③ effect of penehyclidine hydrochloride on recurrence of the CPP effect induced by morphine injection. Mice were randomly divided into control, morphine-dependent, and penehyclidine hydrochloride groups (n = 18).

Effects of Penehyclidine Hydrochloride on Morphine Induced CPP

A pre-experiment was conducted to test the natural environmental preference of mice. The mice were allowed to move freely in the shuttle box for 15 min once daily for 3 consecutive days. On day 4, the average time spent in the gray and black zones was calculated to determine the natural environment preference of the mice. (2) The two zones were completely separated by a separated partition to establish the morphine induced CPP effect. On the 5th day of the experiment, mice in the morphine-dependent and penehyclidine hydrochloride groups were injected subcutaneously (sc) with 5 mg/kg of morphine at 9:00 am, then placed in the gray area for 50 min. After the mice were injected sc with the same volume of normal saline at 4:00 PM, mice were placed in the black area for 50 min. The mice in the control group were injected with the same volume of normal saline the last afternoon. The patients were trained for 8 consecutive days. (3) To determine the effect of Penehyclidine hydrochloride on morphine induced CPP, morphine was discontinued on day 13, and 6 mice were randomly selected from each group. Penehyclidine hydrochloride group Mice in the Penehyclidine hydrochloride (0.5 mg/kg) and control groups received an intraperitoneal (ip) injection of ether hydrochloride. Mice in the morphine-dependent group received an ip injection of physiologic saline. Clapboard transposition into the channel-type clapboard was done 30 min after the CPP test, and mice were observed in the grey area within 15 min. After the test, the mice were sacrificed, the brain were removed and placed in liquid nitrogen for cryopreservation, a refrigerator at - 80 °C.

The CPP effect induced by morphine subsided on the 14th day, and the mice were injected with the same volume of normal saline in the morning and moved to the gray and black areas for 50 min. The CPP effect subsided after 10 days. On the 24th day, 6 mice were randomly selected from each group for CPP testing using a channel-type partition, and the residence time of mice in the gray zone within 15 min was observed. At the end of the test, the mice were sacrificed, and the brains were removed, then frozen in liquid nitrogen and stored in a -80 °C refrigerator for later use.

The effect of penehyclidine hydrochloride on the reappearance of CPP induced by morphine injection was determined. On day 25, mice in the PHC group mice were injected with PHC (0.5 mg/kg). The mice in the control and morphine-dependent groups were injected ip with the same volume of normal saline. After 30 min, the 3 groups of mice were injected with morphine (1 mg/kg), the channel-type partition was used, then tested for the CPP effect. The mice in the penehyclidine hydrochloride group were observed for the CPP effect induced by morphine injection again. After the test, the remaining 6 mice were sacrificed and the brains were removed, cryopreserved in liquid nitrogen and placed in a refrigerator at -80 °C refrigerator.

After the end of each stage of the CPP experiment, the mice were sacrificed under anesthesia. According to the stereotaxic map of the mouse brain, the VTA, NAc, and PFC were quickly separated and stored in a -80 °C refrigerator for later use. The total protein was extracted from the RIPA lysate containing a protease inhibitor, and the concentration of total protein was measured using the BCA method [8]. SDS-page was performed on 50 μg of each sample. The samples were transferred to PVDF membranes and sealed for 1 h. The samples were incubated with primary antibodies (anti-*β*-arrestin-2 and anti-*β*-actin) overnight at 4 °C, then incubated with the secondary antibody at room temperature for 1 h. The membranes were thrice-washed with 1×TBST for 10 min each time. The integrated absorbance value of protein bands was analyzed by a Genesnap imaging analysis system (Alpha Ease FC software, Protein Simple company, Silicon Valley, The USA). The light density scanning method was used for one-half quantitative analysis of the development zone. Groups of related brain region *β*-arrestin-2 expression and *β*-arrestin-2: *β*-actin ratio of the optical density value.

Using SPSS 20. 0 software for data statistics processing, measurement data were presented. The in the grey area residence time of mice and various brain region *β*-arrestin-2 expression were evaluated with single-factor analysis of variance. The least significant difference (LSD) method was adopted to evaluate two groups. A $P<0.05$ was considered statistically significant.

RESULTS

Table 1: Gray zone residence time of mice in three groups at each stage (n=6 per group, in seconds).

Group	Morphine Induced CPP	CPP Fade	Morphine Kindling Induces Reappearance of CPP
The control group	347 ± 83	386 ± 97	369 ± 98
Morphine Group	676 ± 115a	403 ± 93	455 ± 89c
Penehyclidine hydrochloride group	579 ± 107b	449 ± 91	428 ± 95d
F	13.264	14.244	15.454
P	< 0.01	0.122	< 0.05

Note: In the CPP stage induced by morphine, compared with the control group, $^{a}P<0.05$; Compared with morphine dependent group, $^{b}P<0.05$. In the CPP reappearance stage, compared with the control group, $^{c}P<0.05$; Compared with morphine dependent group, $^{d}P<0.05$.

Preliminary experimental results showed that mice in the black and grey zones had average residence times of (611+98) s and (289+98) s, respectively. The black area was a natural preference area of mice compared to the grey area as defined by the with medicine area. As shown in Table 1, 8 days after morphine training single factor analysis of variance showed that the comparison group difference was statistically significant [$F(2,2) = 13.264$, $P<0.01$].

Compared with the control group, the morphine-dependence group grey area residence time was higher (P<0.05). Compared with the morphine-dependence group, Penehyclidine hydrochloride group grey area had lower residence time, the difference was statistically significant (P<0.05). After 10 days elapsed, single factor analysis of variance showed that among the three groups there was no statistically significant difference [F (2,2)=14.244, P> 0. 05] as shown in Table 1.

After morphine injection, single factor variance analysis revealed a comparison group difference was statistically significant [F (2, 2) = 15.454, P<0. 05] as shown in Table 1. Compared with the control group, the morphine-dependence group grey area residence time was higher; the difference was statistically significant (P<0.05). Compared with the morphine-dependence group, Penehyclidine hydrochloride group grey area low residence time; the difference was statistically significant (P<0.05).

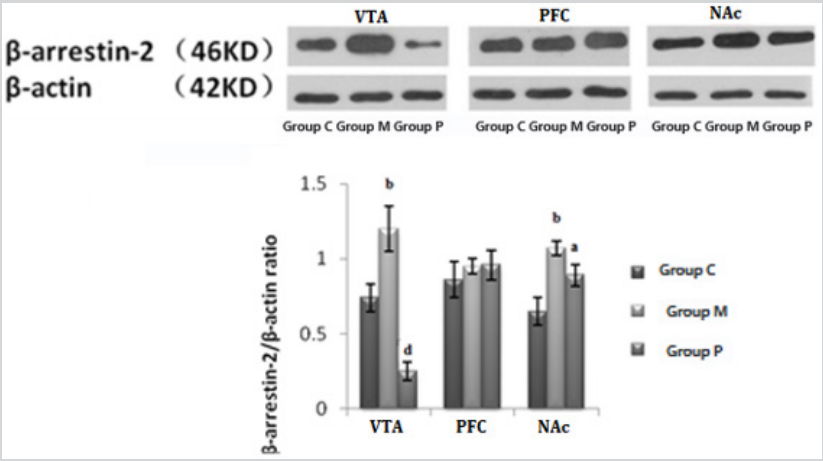

Figure 2: Changes in β-arrestin-2 protein expression in relevant brain regions of mice in each group after CPP establishment Comparison with control group ^{b}P 0.01; Compared with morphine dependent group ^{a}P<0.05, ^{d}P<0.01.

Note: VTA shows the ventral tegmental area of midbrain. PFC shows prefrontal cortex. NAc, nucleus accumbens. Group C shows control group, group M shows morphine dependent group, Group P shows penehyclidine hydrochloride group.

Three groups of mice had related brain regions in different stages of the β-arrestin-2 protein expression. After 8 days of morphine training, 3 groups of mice had VTA brain region β-arrestin-2 expression; the difference was statistically significant [F(2, 2) = 16.841, P<0. 05] as shown in Figure 2. Compared with the control group, the morphine-dependent group had high β-arrestin-2 expression (bP<0.01). Compared with the morphine-dependence group, Penehyclidine hydrochloride group had lower β-arrestin-2 expression (dP<0.01). NAc β-arrestin-2 expression exhibited a similar phenomenon; the difference was statistically significant [F(2, 2)=18.032, P<0. 05]. Three groups of mice in PFC β-arrestin-2 levels had no significant difference.

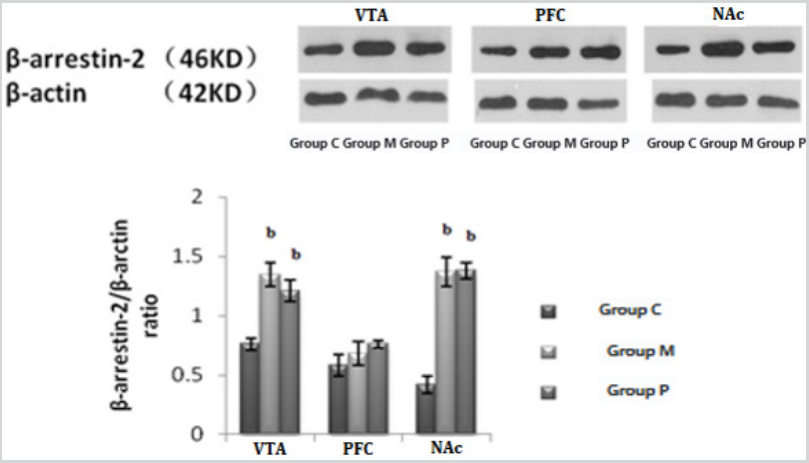

Figure 3: Changes in β-arrestin-2 protein expression in relevant brain regions of mice in each group after CPP resolution Comparison with control group ^{b}P <0.01.

Note: VTA shows the ventral tegmental area of midbrain. PFC shows prefrontal cortex. NAc, nucleus accumbens. Group C shows control group, group M shows morphine dependent group, Group P shows penehyclidine hydrochloride group.

After 10 days had elapsed, 3 groups of mice in the VTA brain region β-arrestin-2 protein expression; the difference was statistically significant [F(2, 2) = 18.625, P<0. 05] as shown in Figure 3. Compared with the control group, the morphine-dependent group Penehyclidine hydrochloride group had high β-arrestin-2 expression (both bP < 0.01). The level of β-arrestin-2 in NAc also

showed a similar phenomenon; the difference was statistically significant [F(2,15) = 17.952, *P*<0.05]. Three groups of mice in PFC *β*-arrestin-2 levels had no significant difference. After morphine ignites, three groups of mice VTA brain regions *β*-arrestin-2 protein expression; the difference was statistically significant [F (2, 2) = 18.492, *P*<0. 05] as shown in Figure 4. Compared with the morphine-dependence group, the control and Penehyclidine hydrochloride group *β*-arrestin-2 expression quantity was lower (a*P*< 0.01, d*P*<0.05). NAc in *β*-arrestin-2 levels also had a similar phenomenon; the difference was statistically significant [F(2, 2)=19.214, *P*<0. 05]. *β*-arrestin-2 protein expression in PFC; the difference was statistically significant [F (2, 2)=20.131, *P*<0.05]. Compared with the control group, the penehyclidine hydrochloride group *β*-arrestin-2 expression quantity group were higher (b*P*<0.01), compared with the morphine-dependence and control groups *β*-arrestin-2 expression quantity was lower (a*P*<0.01).

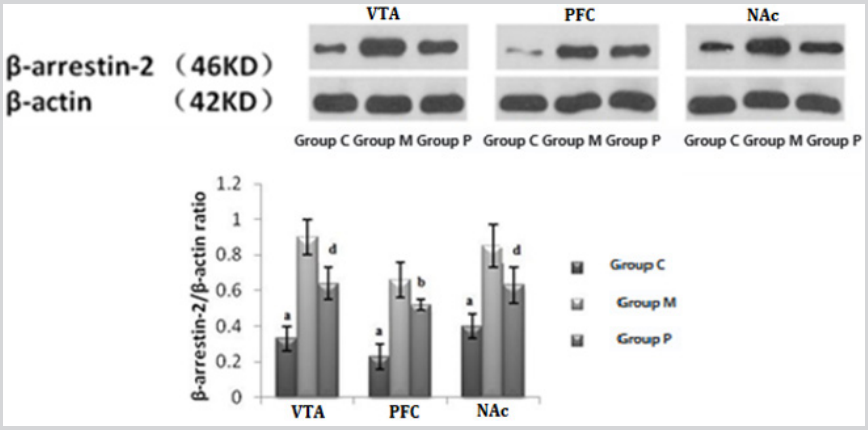

Figure 4: hanges in β-arrestin-2 protein expression in relevant brain regions of mice in each group after CPP reappearance Comparison with control group ^{b}P<0.01; Compared with morphine dependent group aP <0.05, ^{d}P <0.01.

Note: VTA shows the ventral tegmental area of midbrain. PFC shows prefrontal cortex. NAc, nucleus accumbens. Group C shows control group, group M shows morphine dependent group, Group P shows penehyclidine hydrochloride group.

DISCUSSION

CPP building, fading, and building again is a state of relapse and degree of efficacy in animal models. The CPP animal model can cause relapse behavior of the injected exciter, including training, environmental signals, and stress; training drugs are the most effective stimulus. About morphine training has subsided, CPP maintains time and different laboratory results [10-13]. It has been reported that *β*-arrestins are widely expressed in mammalian brain and have obvious differences in expression in brain regions [14]. The expression of *β*-arrestin-2 in rat brain is much higher than *β*-arrestin-1, especially in the hippocampus, hypothalamus, and striatum [14-16]. Both β-arrestins are significantly distributed in synaptic endings, suggesting that *β*-arrestins may have important roles in the nervous system. The intensity and duration of analgesia in *β*-arrestin-2 knockout mice induced by a single morphine injection were significantly higher than wild-type mice [18,19]. Moreover, the gene knockout mice do not have tolerance to repeated morphine injections [17,19]. All the results suggest that *β*-arrestin-2 has an important role in the development of drug-induced psych dependence. Therefore, for the treatment of opioid dependence, selective downregulation of *β*-arrestin-2 is one of the development directions of future treatment measures. Studies have shown that morphine causes changes in the dopamine level in the striatum of *β*-arrestin-2 knockout mice and affects the CPP effect of morphine dependence [19-21] suggesting that *β*-arrestin-2 may be involved in the formation of drug dependence.

Studies have shown that penehyclidine hydrochloride can selectively act on M1 and M3 receptors, and quickly cross the blood-brain barrier and enter the brain, which has a certain central sedation effect [21-25]. Jianzhong Qiao [21] studied the pharmacokinetic characteristics of penehyclidine hydrochloride in mice and found that the absorption and distribution of penehyclidine hydrochloride reached a peak quickly. On the basis of pharmacokinetics, our group conducted pre-experiments to explore the optimal dose of penehyclidine hydrochloride, and finally selected 3 doses of 0.5 mg/kg for the study. The results showed that acute treatment with penehyclidine hydrochloride during the dependence and relapse stage of morphine effectively inhibited the expression of *β*-arrestin-2 in related brain regions of morphine-dependent mice, indicating that penehyclidine hydrochloride effectively inhibited the formation of long-term memory of morphine dependence by reducing the expression of *β*-arrestin-2 in related brain regions of morphine-dependent mice. A previous study from our group also found that penehyclidine hydrochloride inhibited morphine relapse [19-20,26].

The aim of this study was to establish a full-stage model of the establishment, regression, and recurrence of conditional CPP induced by morphine in mice, and to observe the effect of PHC on the expression of *β*-arrestin-2 in the VTA, PFC, and NAc during the development of morphine-induced mental dependence. By using a video tracking CPP model system, we established a full-stage model of CPP formation, regression, and reactivation in morphine-dependent mice, and dynamically detected the changes in *β*-arrestin-2 expression in the VTA, PFC, NAc, and other brain regions. In elucidating the role of *β*-arrestin-2 in different brain regions in different stages of morphine dependence, we found that the levels of *β*-arrestin-2 in the VTA and NAc in morphine-dependent mice were significantly higher than the control group during the formation, regression, and relapse of morphine dependence, and the levels of *β*-arrestin-2 in the PFC were higher than the control group during the relapse stage.

CONCLUSION

This finding suggests that the VTA and NAc play an important role in the formation, regression, and reactivation of morphine dependence in mice, while the PFC mainly plays an important role in the relapse stage of addiction through regulating β-arrestin-2 protein. The main shortcoming of this paper was that the adaptive changes of central β-arrestin-2 and the molecular mechanism of PHC action during the formation of opioid mental dependence are not clear. We will further this issue in a corollary study.

DECLARATIONS

Ethics Approval and Consent to Participate: All experimental procedures were approved by the Animal Ethics Committee of Zhongnan Hospital and Research Centre and Animal Ethics Committee of Wuhan University, Hubei, China, and performed in accordance with the National Institutes of Health "Guidelines for the Care and Use of Laboratory Animals". The study was carried out in compliance with the ARRIVE guidelines.

Availability of Data and Material: The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Authors' Contributions: Jiuhong Ye Xiao-bo Feng Yu-feng Zou and Ruiwen Ding finished most of the work of the experiments. Kai Chen and Jiuhong Ye wrote the manuscript. Xiao-bo Feng Yu-feng Zou and Ruiwen Ding was responsible for data management. Kai Chen and Xiao-bo Feng was responsible for the original design and provided critical revisions. Kai Chen and Jiuhong Ye carried out the statistical analysis and interpretation. All authors read, contributed to and approved the final manuscript.

Authors' Information: Jiuhong Ye is the deputy director of public health, deputy chief nurse at Zhongnan Hospital of Wuhan University. Kai Chen and Ruiwen Ding are the attending physician in the department of anesthesiology at Zhongnan Hospital of Wuhan University. Yufeng Zou is a resident physician in the department of anesthesiology at Zhongnan Hospital of Wuhan University. Xiao-bo feng is an associate chief physician in the department of anesthesiology at Zhongnan Hospital of Wuhan University.

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