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Research paper

# Epigenetic upregulation of CXCL12 expression contributes to the acquisition and maintenance of morphine-induced conditioned place preference

Huan Liu[a,b,1], Jiayou Wei[a,b,1], Meng Liu[a,b,1], Shaoling Wu[c], Chao Ma[c], Cuicui Liu[c], Kangmei Huang[d], Xueqin Zhang[d], Ruixian Guo[a,b], Kuibo Zhang[a], Wenjun Xin[a,b,*]

[a] Neuroscience Program, Zhongshan School of Medicine, Fifth Affiliated Hospital of Sun Yat-sen University, Sun Yat-sen University, Guangdong, PR China
[b] Guangdong Province Key Laboratory of Brain Function and Disease, Department of Physiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangdong, China
[c] Department of Rehabilitation Medicine, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou 510120, PR China
[d] Department of Psychology, Key Laboratory of Neurogenetics and Channelopathies of Guangdong Province and the Ministry of Education of China, Guangzhou Medical University, 195 Dongfeng West Road, Guangzhou 510182, PR China

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ABSTRACT

Addiction and rewarding effect is a primary side effect of morphine, which is commonly used to relieve the acute or chronic pain. Several lines of evidence have suggested that inflammation response in the VTA contributes to morphine-induced reward (conditioned place preference, CPP), while the mechanism are poorly understood. The present study showed that repeated morphine conditioning persistently increased the expression of CXCL12 mRNA and protein in VTA. Furthermore, inhibition of CXCL12 prevented the acquisition and maintenance, but not the expression, of morphine-induced CPP in rodent. In addition, molecular analysis revealed that morphine conditioning increased the occupancy of p-STAT3 in the specific binding site (-1667/-1685) of CXCL12 promoter regions, and enhanced the interaction between acetyltransferase p300 and STAT3, and, hence, induced the histone H4 hyperacetylation in the promoter region and facilitated the transcription and expression of CXCL12 in VTA. Collectively, these results, for the first time, provided the evidence that persisted increase of VTA CXCL12 via epigenetic mechanism mediated the acquisition and maintenance, but not the expression, of morphine CPP.

## 1. Introduction

Although opioids such as morphine and its derivatives have been the mainstay of therapies for clinical pain control over the past decades, they also induce severe adverse side effects, including tolerance, dependence and addiction (Ballantyne and LaForge, 2007; Woolf and Hashmi, 2004). Accumulating evidence showed that acute and chronic treatment with morphine induced a central proinflammatory response that contributes to the development of morphine addiction-related behavior (Eidson and Murphy, 2013). Morphine-induced neuroinflammation is evidenced by the existence of glial cell activation and proinflammatory cytokines release in CNS (Ninkovic and Roy, 2013). Inhibition of glial cell activation in NAc prevented morphine-induced reward effect (Zhang et al., 2012). Similarly, cytokines such as TNF-α or IL-6 in CNS are also emerging as the important modulators of addiction-related behavior (Nakajima et al., 2004; Zalcman et al., 1999). These results suggested that inflammatory responses in VTA or NAc played an important role in the reward effect of morphine.

The chemokine CXCL12 (also known as stromal cell-derived factor 1; SDF1) has been found to play important roles in several processes involved in ischemic stroke (Wang et al., 2012), brain tumor pathogenesis (Duda et al., 2011) and multiple sclerosis (Carbajal et al., 2010). Interestingly, CXCL12 is constitutively expressed by mesencephalic DA neurons (Banisadr et al., 2003), and the upregulation of CXCL12 can directly enhance the excitability of substantia nigra DA neurons by increasing the amplitude of high voltage-activated N-type calcium currents. (Skrzydelski et al., 2007). In addition, CXCL12 has also been shown to regulate morphine-induced tactile hyperalgesia (White and Wilson, 2008). However, whether chemokine CXCL12 in the VTA mediates the acquisition and maintenance of reward effect following morphine treatment remains unclear.

It has been suggested that the induction of CXCL12 expression was mediated via activation of several protein kinases or transcription factors, such as p38 MAPK, NF-κB and transcription factors activator protein 1 (AP-1) (Hsieh et al., 2014; Sung et al., 2009). Once activated, STAT3, a transcription factor that belongs to the STAT family, is translocated to the nucleus where it binds to the DNA and regulates the transcription and expression of inflammation-associated genes (Nicolas

* Corresponding author at: Zhongshan Medical School, Sun Yat-Sen University, 74 Zhongshan Road 2, Guangzhou 510080, PR China.
*E-mail address:* xinwj@mail.sysu.edu.cn (W. Xin).
[1] These authors contributed equally to this work.

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et al., 2013), which might be involved in the addictive behaviors of abuse drugs (Hong et al., 2016). Activated STAT3 can recruit several types of histone acetyltransferases to the promoter region of target genes and facilitate the expression of target genes by changing the acetylation profiles of histones (Reich and Liu, 2006; Yu et al., 2009). Histone acetylation has been recognized as an important factor to modify the accessibility of the DNA to the transcriptional machinery. Our previous studies have demonstrated that STAT3 in the VTA plays a critical role in the reward effect of morphine (Chen et al., 2017). Hence we hypothesize that chronic morphine induces STAT3 activation and, consequently, mediates CXCL12 upregulation, which contributes to the reward effect of morphine.

## 2. Materials and methods

### 2.1. Animals

Male Sprague-Dawley rats weighing 200 to 250 g were purchased from the Institute of Experimental Animals of Sun Yat-Sen University. *CXCL12*$^{-/+}$ heterozygotes mice and *STAT3*$^{flox/flox}$ mice were purchased from the Jackson Laboratory. All animals were housed in separated cages and the room was kept at 24 °C temperature and 50–60% humidity, under a 12/12 h light/dark cycle and with ad libitum access to food and water. All experimental procedures were approved by the Local Animal Care Committee and were carried out in accordance with the guidelines of the National Institutes of Health on animal care and the ethical guidelines.

### 2.2. Surgery and infusion procedures

The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and permanent guide cannulae (23 gauge, RWD Life Science, Shenzhen, China) were implanted bilaterally 1 mm above the VTA DA neurons area (Fig. 1A). The stereotaxic coordinates for VTA of rats were as follows: AP: −5.8 mm; ML: ± 2.3 mm; DV: −8.4 mm; angle 10° (Paxinos & Watson, 1997). The cannula was secured by dental cement and anchored to stainless steel screws fixed to the skull. To prevent clogging and infection, a stainless steel obturator was inserted into the guide cannula. The animals were allowed to recover from surgery for 6 days. Drugs or saline (vehicle) were delivered through an injector needle, which was terminated 1 mm below the tip of the guide cannula, at a continuous rate of 0.1 μl/min under the control of a micro-infusion pump. Injector needles remained in place for 4–5 min before being pulled out to allow the drugs to completely diffuse from the tips, and then the obturator was reinserted into the guide cannula. Totally 37 rats were excluded from the final analysis due to the misplaced cannula. CXCL12 neutralizing antibody, STAT3 activity inhibitor S3I-201 or CXCL12 siRNA were bilaterally microinjected into VTA 15 min prior to each morphine treatment.

### 2.3. Conditioned place preference (CPP)

The CPP apparatus was used as previously described (Guo et al., 2016; Zhang et al., 2012). Briefly, the test apparatus consisted of a shuttle-box that was divided into two compartments of same size ($30 \times 30 \times 53\ cm^3$) by a central partition (1 cm thick) with a door. One compartment was white with a grid floor and the other compartment was black with a smooth floor. To minimize the effect of novelty and stress associated with exposure to the apparatus during morphine CPP, rats were habituated to the apparatus for 15 min for three consecutive days before the pre-conditioning test. In the pre-conditioning test (day 0), animals were placed in the CPP apparatus and the door was removed to allow animals for free access between both chambers for 15 min. The time spent in each compartment was recorded. In our experiment, most of rats exhibited natural preference for the black compartment (preferred side). Therefore, the biased CPP procedure used in our present study, we selected the white compartment (non-preferred side) as the morphine-paired side and the black compartment as the saline-paired side. The animals showing strong unconditioned preference (> 800 s) were discarded. During conditioning (day 1–5), rats were firstly injected with saline subcutaneously in the morning and immediately confined to the black compartment for 30 min; about 6 h later, the rats were subcutaneously injected with morphine and immediately placed in the white compartment for 30 min. On the next day, the animals received morphine administration in the morning and saline injection in the afternoon. On the third and fifth day of conditioning, the same schedule as the first day was repeated. The schedule on the fourth day was the same as the secondary day. For the CPP test on day 6, rats were allowed to move freely in the test box for 15 min and the time spent in the white compartment was recorded for each animal. The change of preference was calculated as the difference (in s) of the time spent in the drug-paired compartment (white compartment) between the postconditioning day and the preconditioning session.

#### 2.3.1. Acquisition experiment

Drug or vehicle was injected into the VTA 30 min prior to each morphine administration during conditioning. CPP was evaluated on day 6 of the experiment as indicated in Fig. 1B (CPP Test 1). To exclude the potential effect of inhibitors themselves on the conditioning, drug alone was micro-injected into the VTA in the animals conditioned with saline.

#### 2.3.2. Expression experiment

Following the acquisition of CPP, the rats received a single intra-VTA injection of drug in the home cage on day 6, and CPP was tested 30 min later.

#### 2.3.3. Maintenance experiment

Following the acquisition of CPP, the rats were given daily intra-VTA injection of drug in the home cage for another 5 days (day 6–10). On day 11, the rats were tested for CPP (CPP Test 2, Fig. 1B).

### 2.4. RNA extraction and real-time quantitative PCR

The reverse transcription was performed with oligo-dT primer and M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's protocol. The sequences of specific primers for the examined mRNA and the internal standard for PCR reactions were listed in Table 1A. The relative expression ratio of mRNA in the VTA tissues was quantified by the $2^{-\Delta\Delta CT}$ method.

### 2.5. Western blot

The animals were immediately anesthetized with sodium pentobarbital (50 mg/kg, i.p.) after the CPP paradigm was performed. The brains were removed and sectioned in a cryostat for the VTA (−4.9 to −5.8 mm relative to the bregma). The areas of VTA was strictly punched with a 15-gauge cannula on ice and sonicated on ice in 15 mmol/l Tris buffer containing a cocktail of proteinase and phosphatase inhibitors. The supernatants samples were separated by gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody following incubation of primary antibody. The band was quantified with computer-assisted imaging analysis system (NIH Image J).

### 2.6. Immunohistochemistry

Animals were immediately anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused through the ascending aorta with cold heparinized saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2–7.4). The brain was removed, post-fixed for 3 h in