Naloxone-precipitated withdrawal enhances ERK phosphorylation in prefrontal association cortex and accumbens nucleus of morphine-dependent mice

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Abstract

Mitogen-activated protein kinases (MAPK) can be activated by opioids such as morphine via opioid receptor, and their activations have been observed in synaptic plasticity, learning, memory and addiction. Long-term exposure to morphine may induce physical dependence, manifested as somatic withdrawal symptoms such as diarrhea, body weight loss, jumping and headshaking, when drug is deprived. Though morphine dependence and withdrawal have been extensively studied, their molecular mechanisms have not been fully elucidated. In the present study, the physical dependence on morphine was developed in mice by an intermittent, escalating procedure of morphine injections, and was measured by the body weight loss and the behavioral signs (jumping and headshaking). We found that the mice with chronic morphine administration experienced dramatic body weight loss, compared with the saline-treated controls. Naloxone-precipitated withdrawal led to more body weight loss, compared with spontaneous withdrawal. Naloxone-precipitated withdrawal mice showed significantly aggravated morphine-withdrawal symptoms (including jumping and headshaking), compared with spontaneous withdrawal mice. MAPK pathway activities in the frontal association cortex (FrA), accumbens nucleus (Acb) and caudate putamen (CPu) were examined to probe into molecular mechanism for morphine dependence and withdrawal. Compared with saline-treated mice, morphine-dependent mice and spontaneous withdrawal mice, naloxone-precipitated withdrawal mice showed a significantly increased ERK phosphorylation in FrA and Acb, but not in CPu. However, the activities of other protein kinases in the MAPK pathway, including p38 and JNK, showed no changes in FrA, Acb and CPu of the mice during the chronic morphine dependence and withdrawal phases. These results suggest that the ERK phosphorylation in FrA and Acb may be associated with naloxone-precipitated withdrawal syndrome.

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A R T I C L E   I N F O

Article history:
Received 28 August 2009
Received in revised form 7 November 2009
Accepted 9 November 2009

Keywords:
Phenomenon
Dependence
Withdrawal syndrome
Frontal association cortex (FrA)
Accumbens nucleus (Acb)
Caudate putamen (CPu)
MAPK pathway

A B S T R A C T

Drug addiction has been widely accepted as a chronic relapsing brain disease [14] and a disease of learning and memory [28]. Repeated exposures to drugs will change the neuron plasticity in different regions of brain and further lead to the behavioral abnormalities associated with cognitive deficits, tolerance and dependence [23,27]. Previous studies have shown that mitogen-activated protein kinases (MAPKs) are closely related to the behavioral abnormalities induced by addictions to various drugs such as cocaine, amphetamine and morphine [9]. In mammals, three major MAPK pathways have been identified: MAPK/ERK, SAPK/JNK and p38 MAPK. Chronic morphine treatment has been shown to increase MAPK activities in whole brain [20]. However, little is known about the exact brain areas where MAPKs are active during morphine dependence and withdrawal. A great amount of evidence has suggested that the spinal cord is the crucial site where opioid withdrawal is mediated [3]. Several brain structures, including the frontal association cortex (FrA), accumbens nucleus (Acb) and caudate putamen (CPu), have also been found to be involved in the neural circuitry underlying drug-seeking behavior [4,18]. Our previous study has shown that FrA plays an important role in morphine craving and that ERK2 is involved in eliciting the environment-related morphine craving [15]. Based on these findings, we hypothesized that MAPK/ERK, SAPK/JNK and p38 MAPK are involved in morphine dependence and morphine withdrawal-evoked neural plasticity in FrA, Acb and CPu of the mouse forebrain.

In this study, we used an intermittent, escalating dosing schedule of morphine administration to develop morphine-induced...
physical dependence in mice. Jumping and head shaking, the typical withdrawal behaviors, were used to test the degree of physical dependence on morphine. MAPK pathway activities in FrA, Acb and CPu were examined to explore the molecular mechanism for the morphine dependence and withdrawal in mice.

Animals: Male C57BL/6 mice (8 weeks old, 25–30 g) were housed in groups of four per cage at constant temperature (20–22 °C) and humidity (50–55%) under a 12 h light/dark cycle with food and water provided ad libitum. Mice were allowed to habituate in the colony room for 1 week before experimental manipulations were undertaken. All animal protocols used were approved by the Animal Care and Use Committee of Xi’an Jiaotong University.

Drug and drug treatment protocols: 32 male C57BL/6 mice were randomly divided into four equal groups: chronic morphine treatment group, saline-treated group, spontaneous morphine-withdrawal group and naloxone-precipitated withdrawal group. Morphine HCl (the First Pharmaceutical Factory of Shenyang, China) and naloxone HCl (Huasu Pharmaceutical Factory of Beijing, China) were dissolved in 0.9% sodium chloride solution, respectively. Then, 24 mice were injected intraperitoneally (i.p.) with the morphine solution at a volume of 10 ml/kg body weight. The other 8 mice were administered with an equivalent volume of saline. Chronic morphine dependence in mice was developed according to the reported treatment schedule [11,5]. The 24 mice were administered with gradually increasing doses of morphine twice daily (at 9:00 and 18:00) for 7 consecutive days (day 1: 10 and 10 mg/kg; day 2: 20 and 20 mg/kg; day 3 and day 4: 40 and 40 mg/kg; day 5: 60 and 60 mg/kg; day 6: 80 and 80 mg/kg; day 7: 100 and 100 mg/kg). 2 h after the last dose of morphine was given, the mice in the chronic morphine treatment group were weighed and sacrificed and those in the naloxone-precipitated withdrawal group were administered with naloxone (2 mg/kg, i.p.) to induce withdrawal behaviors. The withdrawal behaviors of the spontaneous morphine-withdrawal group were monitored 24 h after the last administration of morphine. The behavioral signs of withdrawal syndrome were measured by the body weight loss and the number of jumping and head shaking within 30 min. Brain tissues from FrA, Acb and CPu were dissected on ice, frozen immediately in liquid nitrogen and stored at −80 °C for later use.

Sample preparation and immunoblotting: Brain tissue samples from FrA, Acb and CPu were homogenized in 50 mM RIPA buffer (20 μl/mg), containing protease inhibitor cocktail and phosphatase inhibitor cocktail. Homogenates were incubated on ice for 30 min and centrifuged at 12,000 g at 4 °C until use. Then, the samples, containing 20 mg of total protein, were denatured at 95 °C for 5 min, separated by 12% SDS-PAGE and transferred to 0.45 μm nitrocellulose membranes. The membranes were blocked in 5% nonfat dry milk with TTBS for 1 h at room temperature. The membranes were successively incubated with primary antibodies pERK, pJNK and pP38 (Cell Signaling Co.), gently agitated overnight at 4 °C, then incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) and developed by using enhanced chemiluminescence (ECL). All western blot analyses were performed at least three times and parallel results were obtained.

Quantitation of MAPK immunoreactivity: The autoradiogram bands of the MAPK proteins (pERK, pJNK, pP38) in FrA, Acb and CPu were scanned in the transmittance mode at a resolution of 600 dpi using a Scansaker9700dxl scanner (China). The scanned images were analyzed using Image-Pro Plus image program. The integrated optical densities (IOD) of protein bands were compared to reveal their expressions and activities.

Statistical analysis: The data were expressed as means ± S.E. Statistical significance was assessed by one-way analysis of variance (ANOVA) and post hoc individual comparisons were made using the paired samples t-tests. The significance level was set at p < 0.05. The chronic morphine treatment group, the naloxone-precipitated withdrawal group and the spontaneous morphine-withdrawal group showed marked body weight loss, compared with the saline group. The naloxone-precipitated withdrawal group lost the most body weight (p < 0.05) (Fig. 1). However, body weight loss did not show much difference between the chronic morphine treatment group and the spontaneous morphine-withdrawal group (p > 0.05).

As shown in Fig. 2, following repeated administrations of morphine, mice that had received naloxone injection showed dramatically increased signs of withdrawal, which were manifested by jumping and head shaking, compared to the spontaneous morphine-withdrawal group (p < 0.05). Therefore, naloxone tended to enhance withdrawal syndrome in morphine-dependent mice.
To understand the molecular mechanism for the behavioral changes in mice with morphine dependence and withdrawal syndromes, western blotting of pERK, pJNK and pP38 MAPK proteins was performed to detect MAPK family activities in different forebrain regions of mice. Compared with the saline-treated group, the naloxone-precipitated withdrawal group showed a significant enhancement in phosphorylated ERK1/2 expression in FrA and Acb (p < 0.05), but not in Cpu (p > 0.05). However, the mice with chronic morphine dependence and withdrawal showed no change in the pP38 and pJNK MAPKK protein levels in FrA, Acb and Cpu, compared with the saline-treated group (p > 0.05) (Fig. 3).

To evaluate the effect of naloxone treatment (alone) on ERK phosphorylation, 16 mice were injected intraperitoneally (i.p.) with saline twice daily for 7 consecutive days. 2 h after the last injection, half of the mice were injected with naloxone (2 mg/kg, i.p.) and the other mice were injected with the same volume of saline. 30 min after the injection, the brains of all the mice were rapidly dissected and FrA and Acb were collected for the ERK phosphorylation detection. As shown in Fig. 4, the naloxone-treated and saline-treated mice showed no difference in the pERK expression in FrA and Acb. Our results are consistent with those of previous studies [20,1].

Recently, it has been reported that MAPK family can be activated by chronic morphine treatment in the central and peripheral nervous systems [6]. In fact, both acute and chronic morphine administrations, as well as withdrawal after chronic morphine administration have been shown to modulate the levels of activated ERK in specific brain regions. Previous studies have revealed that acute administration of morphine tends to significantly increase the immunodensity of phosphorylated MEK1/2 in the rat cortex and the striatum [1] and to enhance the ERK phosphorylation in the association cortices, locus coeruleus, cingulate and somatosensory of mice [8]. Acute morphine administration has been found to down-regulate phosphorylated ERK1/2 levels in Acb and the central nucleus of the amygdale, but not in Cpu [8,19]. It has also been reported that chronic morphine administration increases ERK phosphorylation in the rat ventral tegmental area (VTA) [2]. Our experiments show that chronic morphine treatment...
Additionally, FrA and Acb in the mouse forebrain may be the important pathways, it was PERK rather than pJNK or pP38 that was associated with the development of antinociceptive tolerance to morphine via opioid receptor endocytosis. Chronic morphine treatment increases P38 MAPK phosphorylation, which is crucial to inflammatory cytokine production and signaling [12]. However, P38 MAPK activation has only been found to occur in spinal microglia [29]. In this study, we detected the P38 MAPK activation in FrA, Acb and Cpu of the mice after chronic morphine withdrawal. Hence, the above-mentioned research on chronic morphine dependence may be associated with the activation of the MAP/ERK kinase pathway in FrA and Acb and that tolerance to the inhibitory effects of morphine on ERK phosphorylation in FrA and Acb is probably developed during the chronic morphine administration.

In our study, the mice in the spontaneous morphine-withdrawal group showed no significant change in ERK phosphorylation in FrA and Acb, and demonstrated a slower and less intense behavioral reaction than the naloxone-precipitated withdrawal mice. Therefore, ERK phosphorylation in FrA and Acb was probably mediated via a naloxone-sensitive mechanism. The possible explanation for the significant increase in ERK phosphorylation in the naloxone-precipitated withdrawal mice may lie in the change in the neuronal activity. It has been reported that naloxone-precipitated withdrawal in chronically morphine-dependent mice results in significantly increased mRNA expression of c-fos, a marker of neuronal activity change, in the frontal cortex, thalamus and other brain areas [10,22]. Nevertheless, Schulz et al. [24] have observed a markedly enhanced activity (phosphorylation) of MAP kinase, the upstream of the ERK, in the locus coeruleus, solitary tract and hypothalamic nuclei of rats, but not in the cortex, after naloxone injection. Edwards’s study has shown that 24 h after withdrawal, the pERK increases dramatically in the prefrontal cortex and shows no change in the accumbens. However, after naltrexone-precipitated withdrawal, the pERK increased dramatically in the prefrontal cortex, accumbens and other brain regions [7]. Obviously, there is a discrepancy between others’ and our results of the changes of ERK phosphorylation in the cortex. The discrepancy may result from the different animal species, brain regions (Edwards et al. used rat prefrontal cortex while we used the mice frontal association cortex), analytical sensitivity and/or experimental schedules.

P38 MAPK responds to environmental stress and its pathway is crucial to inflammatory cytokine production and signaling [12]. Recent studies have shown that P38 can be activated due to the neuropathic pain induced by peripheral inflammation and nerve injury [26,25]. Mace [17] has found that P38 MAPK can be activated by morphine via μ-opioid receptor endocytosis. Chronic morphine treatment increases P38 MAPK phosphorylation, which is associated with the development of antinociceptive tolerance to morphine [16]. However, P38 MAPK activation has only been found to occur in spinal microglia [29]. In this study, we detected the P38 MAPK activation in FrA, Acb and Cpu of the mice after chronic morphine dependence and withdrawal; additionally, there was no difference in the level of P38 MAPK phosphorylation in the three brain regions. Our findings indicate that P38 MAPK in FrA, Acb and Cpu may not be associated in morphine dependence or withdrawal. JNK, a MAPK that is selectively associated with apoptosis, can be activated by various apoptotic stimuli [13]. In Fan’s research, single or chronic morphine injection has been found to induce an increase in the level of JNK3 mRNA in frontal cortex, while no significant change in JNK3 mRNA has been detected in other brain regions such as thalamus, hippocampus and locus coeruleus [21]. However, our study shows that JNK phosphorylation did not change in FrA, Acb and Cpu of the mice with chronic morphine dependence. The inconsistence may result from the possibility that mRNA level does not always reflect the transcriptional regulation of the encoding gene expression at protein level.

In summary, our present study indicates that of the MAPK pathways, it was pERK rather than pJNK or pP38 that was associated with naloxone-precipitated morphine-withdrawal symptoms. Additionally, FrA and Acb in the mouse forebrain may be the important brain regions associated with chronic morphine dependence and withdrawal.

Acknowledgements

This work was supported by National Sciences Foundation of China (NSFC, No. 30672356) to C.-X.Y. and Shaanxi Key Project on Science and Technology (2006K14-8) and Medical Youth Fund (YQN0804) to T.L. We thank Guo-li Yan for thoughtful discussion and manuscript revision.

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351


