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Research article

NMDA receptor dependent changes in c-fos and p-CREB signaling following extinction and reinstatement of morphine place preference

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ABSTRACT

Neural circuitry comprising the ventral tegmental area, nucleus accumbens (NAc), prefrontal cortex (PFC) and hippocampus (HIP) has a main role in reward phenomena. Previous behavioral studies indicated that intracerebroventricular administration of AP5 (NMDA glutamate receptor antagonist) and CNQX (AMPA/kainate glutamate receptor antagonist) during the extinction and before reinstatement of morphine-induced conditioned place preference (CPP) reduced the extinction period and reinstatement of morphine-CPP. Therefore, in the present study, we tried to evaluate the effect of antagonism of NMDA glutamate receptors on the p-CREB/CREB ratio and c-fos expression in the NAc, PFC and HIP during these two phases of morphine-CPP in male adult albino Wistar rats. The p-CREB/CREB ratio and c-fos levels were estimated by Western blot analysis. The results revealed that these two factors decreased by antagonism of NMDA glutamate receptors (different doses of AP5) compared to saline-control group in aforementioned regions. The reduction of molecular markers, especially the p-CREB/CREB ratio, after AP5 administration was more during the extinction period. Therefore, it can be assumed that consolidation and reconsolidation of morphine memory via intra-PFC, −NAc and −HIP NMDA glutamate receptors are in accordance with changes in p-CREB/CREB ratio and c-fos levels.

1. Introduction

Interactions between the dopaminergic and glutamatergic systems may be a main component of neural circuitry of reward, including morphine-induced reward [1]. Dopaminergic neurons in the ventral tegmental area (VTA) which plays a pivotal role in motivation- and reward-related behaviors send their projections to the glutamatergic neurons in the prefrontal cortex (PFC) [2]. Glutamatergic projections from the PFC are found into the VTA, nucleus accumbens (NAc), hippocampus (HIP) and basolateral amygdala which are important in reward and addiction phenomena [2].

In our previous study, we demonstrated that intracerebroventricular (ICV) injection of AP5, a NMDA glutamate receptor antagonist, during the extinction period reduced the extinction period without any effect on the reinstatement of morphine-CPP; while, AP5 administration just before the prime-morphine injection decreased reinstatement of morphine-CPP but the location of the involved NMDA receptors remained unclear [3]. Drug administration modulate gene expression and manipulate the expression and function of transcription agents such as the cAMP response element binding (CREB) protein _a transcription factor and a downstream target of extracellular signal-regulated protein kinase (ERK)_ and c-fos in brain-reward regions like the VTA, NAc, PFC and HIP, which contribute to drug seeking behaviors and reinstatement of drug extinction [4–6]. CREB phosphorylation is facilitated by membrane-bound NMDA glutamate receptors [7]. Also, CREB and c-fos play an important roles as molecular basis in cannabinoid, opioid, and alcohol dependencies [8,9]. In this respect, inhibition of CREB expression by NMDA receptor antagonist in the hippocampus, which has a crucial role in memory acquisition, impaired the memory stabilization and learning [10]. Systemic administration of NMDA receptor antagonist (MK-801) prior to drug administration during conditioning decreased pCREB and fos level in the reward regions [11]. In our previous study, we showed that the ICV administration of AMPA/kainite glutamate ionotropic receptors antagonist (CNQX) during the extinction and before the reinstatement of morphine-induced CPP changed the expression of transcription factors such as CREB and c-fos in the NAc, HIP and PFC demonstrating that these AMPA glutamate receptors in these neural regions involve in mediation rewarding effect of morphine [12]. Here, to study the role of NMDA glutamate receptors in modulation of drug-seeking behaviors, ICV administration of AP5 was done during the

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extinction period and before reinstatement of morphine-induced CPP, and changes in p-CREB/CREB ratio and c-fos expression in the reward-related regions including the NAc, HIP and PFC were studied in the rats.

2. Materials and methods

2.1. Surgical preparation

All used protocols were approved by the ethical committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran. In addition, all the experiments were conducted in accordance with the internationally accepted principles for the Care and Use of laboratory Animals (NIH publication No.80-23, revised in 1996).

Male adult albino Wistar rats (Pasture Institute, Tehran, Iran; n = 55), weighing 200–230 g which were housed in a controlled environmental condition (12-h light/dark cycle; lights on at 7:00 A.M.; temperature 22 ± 2 °C; humidity 60–65%) and had free access to water and food were anaesthetized with intraperitoneal (ip) Ketamine10% (100 mg/kg) and Xylazine 2% (10 mg/kg) (Alfasan, Woerden-Holland). A 23-gauge guide cannula with the length of 8 mm was then implanted into the lateral ventricle according to the rat brain atlas [13]: AP = 0.5 mm caudal to bregma, Lat = 1.6 mm lateral to midline, DV = 4.2 mm ventral from the skull surface (guide cannula was implanted 1 mm above the suitable injection place). The guide cannula was supported by a stainless steel screw and dental acrylic cement. Animals were individually housed and made a 5–7 days recovery from the surgery.

2.2. Conditioning place preference paradigm

2.2.1. Apparatus

The CPP apparatus was made of three compartments including two equal-sized Plexiglas compartments (30 × 30 × 40 cm) which were separated by a door and a third section known as the null section (30 × 15 × 40 cm). The compartments had a difference in their wall strips orientation (vertical vs. horizontal) and for making the tactile difference between them, the smooth and net panel were used for their floors [3].

2.2.2. Conditioned place preference phases

The CPP paradigm consists of three phases including pre-conditioning, conditioning and post-conditioning [3].

2.2.2.1. Pre-conditioning phase. At least 30 min were given to rats for habituation to the test room before starting the experiments. Then, each animal was placed in the start box while the removable door was removed so that the animal had free access to entire compartments for 10-min period. The distance traveled and time spent in each compartment were recorded by a camera and analyzed by the Ethovision software (version 7). The rats which spent more time in one chamber compared to another (≥80%) were excluded from the study.

2.2.2.2. Conditioning phase. The day after the pre-conditioning test, 5 mg/kg morphine [morphine sulfate (Temad, Iran) dissolved in sterile normal saline] was subcutaneously (sc) injected and rats were confined to the morphine-paired compartment for 30 min by closing the removable door of the apparatus. After about 6 h, animals received saline (1 ml/kg; sc) and were placed in another compartment for 30-min period. On the next day, animals received saline in the morning and morphine in the afternoon. The protocol for the third day of conditioning phase was the same as the first day of conditioning.

2.2.2.3. Post-conditioning phase. On the fifth day, the door between two compartments of the CPP box was removed so that the animal had free access to the entire of box. During a 10-min period, the movement of the rat was recorded (cm) and the time spent in the drug-paired compartment minus the time spent in saline-paired one was calculated as the conditioning (CPP) score.

2.3. Extinction and reinstatement of morphine-induced CPP

The day after the post-test, the rats (without morphine injection) were placed in the start box once a day. They were allowed to have free access to all compartments for 10-min period. This procedure was repeated for each rat for the next days until the CPP scores in two consecutive days during the extinction period became equal to those on the pre-conditioning day. Extinction is a form of new learning in which associations between the contexts and events are weakened by exposure to the cues in the absence of those events [3,14]. Reinstatement refers to the recovery of a learned response when a subject is exposed to an unconditioned stimulus (drugs such as morphine) after extinction. In the present study, reinstatement was primed using injection of ineffective dose of morphine (1 mg/kg; sc) after the extinction [3,14]. During the reinstatement test, rats were allowed free access to all compartments. Conditioning scores were recorded during a 10-min period [3,15].

2.4. Experimental design

In the present study, animals in all groups (n = 6-8 in each group) passed the CPP stages; then, they were received the appropriate treatments during the extinction period or reinstatement phase. In the first set of experiments, to study the changes in p-CREB/CREB ratio and c-fos level/expression in the NAc, PFC and HIP, daily ICV injection of 5 μl of AP5 (1, 5 and 25 mM) was done during the extinction phase in three groups. AP5 was used as a NMDA receptor antagonist (Tocris Bioscience, UK) which was diluted in 0.9% normal saline for ICV injection. Microinjections were done by inserting an injection cannula (30-gauge needle; 1 mm longer than guide cannula) into the guide cannula, which was connected to a 5-μl Hamilton syringe by a polyethylene tube (PE-20). In saline-control group (forth group), ICV injection of 5 μl saline (as drug vehicle) was done during the extinction period. In the fifth group, ICV injection of 5 μl of 25 mM AP5 was done before the extinction phase and in the last group, 5 μl of 5 mM AP5 was injected on the reinstatement day before the injection of priming dose of morphine (1 mg/kg; sc).

2.5. Western blot analysis

For investigating the alterations in the c-fos level and p-CREB/CREB ratio, after the reinstatement day, rats were sacrificed and the brains were rapidly removed. The brain was put in a stainless steel brain matrix and then the NAc, HIP, and PFC were immediately dissected out bilaterally according to the rat brain atlas [12] and transferred into the liquid nitrogen to be frozen. Tissues were prepared for western blot analysis. So, all behavioral and molecular data were collected from the same rats.

Samples were collected and tissues were sonicated in 1% sodium dodecyl sulfate buffer in Tris-EDTA, pH 7.4 that contained 1 × protease inhibitor cocktail, 5 mM NaF, and 1 × phosphatase inhibitor cocktail. Samples were boiled for 5 min and centrifuged at 16,100 × *g* for 10 min 12% SDS-PAGE gel electrophoresis was used to separate total proteins; then, they were transferred to polyvinylidene fluoride membranes, and probed with specific antibodies. Immunoreactive polypeptides were detected by chemiluminescence using enhanced ECL reagents. Autoradiography was performed afterwards. After quantification of the results using densitometric scan of films, data analysis was done by Image J, in which integrated density of bands after background subtraction was measured. Individual p-CREB values were divided by their respective CREB values to obtain the p-CREB/CREB ratio for each sample, and c-fos values were divided by their respective ß-actin values