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Research report

Changes in phosphorylation of CREB, ERK, and c-fos induction in rat ventral tegmental area, hippocampus and prefrontal cortex after conditioned place preference induced by chemical stimulation of lateral hypothalamus

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ABSTRACT

Experimental evidence indicates that chemical stimulation of lateral hypothalamus (LH) by carbachol can produce conditioned place preference (CPP) in rats. Several lines of evidence have shown that cAMP-response element binding protein (CREB), extracellular signal-regulated kinase (ERK), and c-fos have pivotal role in CPP induced by drugs of abuse, such as morphine, cocaine, nicotine, and alcohol. Therefore, in the present study, we investigated the changes in phosphorylated-CREB (p-CREB) and -ERK (p-ERK), and c-fos induction within ventral tegmental area (VTA), hippocampus and prefrontal cortex (PFC) after the acquisition of CPP induced by intra-LH administration of carbachol. Animals were unilaterally implanted by cannula into LH. For chemical stimulation of LH, carbachol (250 nmol/0.5 μl saline) was microinjected once each day, during 3-day conditioning phase (acquisition period) of CPP paradigm. After the acquisition period, the brains were removed, and p-CREB and p-ERK, and c-fos induction in the ipsilateral VTA, hippocampus and PFC were measured by Western blot analysis. The results indicated a significant increase in level of phosphorylated CREB ($P < 0.01$) in VTA, and PFC ($P < 0.05$), during LH stimulation-induced CPP, while its level decreased in hippocampus ($P < 0.05$). Also, in aforementioned regions, an increase in c-fos level was observed, but this enhancement in PFC was not significant. Moreover, p-ERK changed in these areas, but not significantly. Our findings suggest that studying the intracellular signals and their changes, such as phosphorylated-CREB, can elucidate a functional relationship between LH and other brain structures involved in reward processing in rats.

1. Introduction

Orexins are released from a small number of neurons clustered in lateral hypothalamus (LH), which have extensive projections throughout brain [53]. The orexin system is now known to play a multiple roles affecting a number of brain functions, including metabolic homeostasis, sleep–wake cycles and drug reward-related behaviors [24,54]. Orexin receptors and orexinergic projections from hypothalamus are localized in regions previously shown to play a role in drug addiction, such as ventral tegmental area (VTA), nucleus accumbens (NAc), substantia nigra, nucleus locus coeruleus, and hippocampus [52].

LH orexin neurons and their projections to VTA involve in formation of associations between environmental cues and drug reward [25]. Orexin/hypocretin-containing neurons in lateral hypothalamus project to VTA, and behavioral studies have suggested that orexin neurons play an important role in motivation, feeding, and adaptive behaviors [6]. Previous studies also indicated that orexin neurons heavily innervate both the dopamine-rich VTA and NAc, the structures that drive behaviors motivated by either food or drug reward. Moreover, orexin receptors are expressed at high levels in these both regions [25]. Furthermore, electrophysiological studies have recently shown that orexins may directly activate VTA dopaminergic neurons [34,43]. Hippocampus is a brain region known to participate in associative processes such as declarative memory [14]. Hippocampus has direct excitatory affection to NAc and can activate dopaminergic neurons of VTA [69]. However, hippocampus is not typically considered an integral component of the reward pathway, but it might be expected to play a significant role in the mechanism leading to development of drug addiction [57]. Furthermore, hippocampus is one of the regions where the LH has projection in [66]. In addition to above-stated areas (VTA and hippocampus), prefrontal cortex (PFC) may serve as a possible common pathway for drug seeking by priming injections of drugs,

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and drug-related cues [9]. There is a reciprocal connection between LH and PFC that leads to LH projection to PFC [33]. VTA also sends a dopaminergic projection to PFC [61], and dopamine (DA) terminals are found often in close proximity to hippocampal terminals on PFC neurons [11]. It seems that in reward processing, there are crucial intracellular signaling changes in brain areas associated with addiction such as the aforementioned regions.

The persistent neuroadaptations to addictive drugs induce changes in the number and efficiency of receptors, signal transduction pathways, gene expression and subsequent component of proteins [17,42,50]. At the molecular level, several lines of evidence have suggested that phosphorylation of cyclic AMP-response element binding protein (CREB) [5,46,59], extracellular signal-regulated kinase (ERK) [16,36,55], and c-fos induction [37,46] are highly involved in many forms of experience-dependent plasticity, such as long-term potentiation (LTP), and play an important role in the rewarding effects of many drugs of abuse, such as nicotine [68], morphine [36], cocaine [28], and alcohol [45]. Nevertheless, there has been no investigation regarding the changes of intracellular molecules in orexin-induced CPP. CPP paradigm has been widely used to evaluate the reinforcement effect of drugs of abuse and especially the psychological dependence [2,41,46,60,64].

Based on our recent study which showed that LH stimulation could solely induce CPP [56], we considered these three areas (VTA, hippocampus and PFC) that are pivotal in the reward circuit, in order to investigate changes in the level of intracellular signal molecules such as p-CREB, p-ERK and c-fos following the development of CPP induced by intra-LH administration of carbachol in rats. Our aim for this study was to evaluate if these three molecules could be an indicator for conditioned place preference induced by LH stimulation in these regions.

2. Materials and methods

2.1. Animals

Forty-two adult male albino Wistar rats (Pasteur Institute, Tehran, Iran) weighing 220–320 g were used in these experiments. Animals were housed in groups of three per cage in a 12/12 h light/dark cycle (light on between 7:00 A.M. and 7:00 P.M.) with free access to chow and tap water. The animals were randomly allocated to different experimental groups. Each animal was used only once. Rats were habituated to their new environment and handled for 1 week before the experimental procedure was started. All experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23, revised 1996) and were approved by the Research and Ethics Committee of Shahid Beheshti University of Medical Sciences.

2.2. Drugs

In the present study, the following agents were used: carbachol (Sigma–Aldrich, USA) was dissolved in normal saline. Antibodies directed against p-CREB, CREB, p-ERK, ERK, c-fos and β-actin were obtained from Cell Signaling Technology, USA. Electrochemiluminescence (ECL) kit was provided from Amersham Bioscience, USA.

2.3. Stereotaxic surgery

Rats were anesthetized by intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg), and placed into stereotaxic device (Stoelting, USA). An incision was made along the midline, the scalp was retracted, and the area surrounding bregma was cleaned and dried. Stainless steel guide cannula was unilaterally implanted in LH. The coordinates for this region were determined by rat brain atlas [47], AP = −3 mm caudal to bregma, Lat = +1.6 mm and DV = −8.8 mm ventral from the skull surface (23-gauge, 12 mm guide cannula was 1 mm above the appropriate injection place). The guide cannula was secured in place using a stainless steel screw anchored to the skull and dental acrylic cement. After the cement was completely dried and hardened, a stainless steel stylet was used to occlude the guide cannula during recovery period. Penicillin-G 200,000 IU/ml (0.2–0.3 ml/rat, single dose, intramuscular) was administered immediately after surgery. Animals were individually housed and allowed to recover for 5–7 days before experiments.

2.4. Drug administration

Microinjections were unilaterally performed by lowering a stainless steel injector cannula (30-gauge needle) with a length of 1 mm longer than the guide cannula into LH. The injector cannula was connected to a 1-μl Hamilton syringe by polyethylene tubing (PE-20), then drug solution or vehicle was unilaterally infused over 60 s and was left for a 60 s extra time, followed by replacement of the obdurator. Different doses of carbachol or saline as its vehicle were slowly administered in a total volume of 0.5 μl/rat over a period of 60 s into LH. Injection needle was left in place for an additional 60 s to facilitate diffusion of drug, and then the stylet was reinserted into the guide cannula. All drug solutions were prepared freshly.

2.5. Behavioral test

2.5.1. Conditioning apparatus and paradigm

A three-compartment CPP apparatus was used in this study [23,39]. The apparatus was made of Plexiglas and two compartments were identical in size (30 cm × 30 cm × 40 cm), but differed in shading and texture. Compartment A was white with black horizontal stripes 2 cm wide on walls and also had a net-like floor. Compartment B was black with vertical white stripes, 2 cm wide and also had a smooth floor. The third compartment, C, was a red tunnel (30 cm × 15 cm × 40 cm). It protruded from the rear of the two large compartments and connected the entrances of them. In this apparatus, rats showed no consistent preference for either large compartments (A and B), which supports our un-biased conditioned place preference paradigm. CPP paradigm took place in five continuous days, which consisted of three distinct phases: pre-conditioning, conditioning and post-conditioning. In pre-conditioning phase, each animal was introduced to the apparatus for 10 min. Conditioning phase consisted of a 3-day schedule of conditioning sessions. In this phase, animals received three trials in which they experienced different doses of carbachol, while confined to one large compartment for 30 min, and three trials in which they experienced the effects of saline while confined to the other large compartment by closing the removable wall. Access to the compartments was blocked on these days (acquisition period). In post-conditioning phase on the fifth day (test day), the partition was removed, and rats could access the entire apparatus. The mean time spent for each rat in both large compartments was recorded by ethovision software (Version 3.1), a video tracking system for automation of behavioral experiments (Noldus Information Technology, the Netherlands). Conditioning score (CPP score) represents the time spent in the reward-paired compartment minus the time spent in the same compartment prior to conditioning (pre-conditioning phase) during a 10-min period. The behavioral data reported here are only from animals in which the placements of cannulae were histologically verified (electronic supplementary figure).

2.5.2. Locomotion tracking apparatus

In order to examine the possible effect of different doses of carbachol or saline microinjected into LH on locomotor activity, animal displacement was recorded using a 3CCD camera (Panasonic Inc., Japan) placed two meters above CPP boxes and locomotion tracking was measured by ethovision software. In these experiments, total distance traveled (cm) was measured on the test day, during a 10-min period, in control and experimental groups.

2.6. Western blot analysis

On the test day following conditioning (acquisition period), the rats were sacrificed. Their brains were dissected out and put onto a glass plate on ice, and then VTA, hippocampus and PFC were collected according to Paxinos and Watson atlas [47]. We used micropunch technique for separating VTA and PFC. Briefly, brains were sectioned in a cryostat in coronal plane of VTA, and 1 mm in depth and 0.5 mm in diameter unilateral tissue punches spanning approximately −4.7 mm relative to bregma [47] were obtained with a 21-gauge stainless steel stylet (Fig. 1A). In the other group of animals, PFC was unilaterally punched 1 mm in depth and diameter from the sagittal plane of brain about 0.4 mm relative to midline [47] with a 17-gauge stainless steel stylet (Fig. 1B). Also, the whole hippocampus was unilaterally dissected from different brains in experimental and control groups. Tissues were sonicated in 1% sodium dodecyl sulfate buffer in Tris–EDTA, pH 7.4, containing 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. Then, the total proteins were electrophoresed in 12% SDS–PAGE gels, transferred to polyvinylidine fluoride membranes and probed with specific antibodies. Immunoreactive polypeptides were detected by chemiluminescence using enhanced ECL reagents and subsequent autoradiography. Quantification of the results was performed by densitometric scan of films. Data analysis was done by Image J., measuring integrated density of bands after background subtraction. Protein concentrations were determined according to Bradford's method [7]. Standard plot was generated using bovine serum albumin.

2.7. Experimental design

2.7.1. Behavioral protocol

To evaluate the dose–response effects of carbachol microinjected into LH on CPP paradigm, carbachol as a LH chemical stimulation agent was established. Different doses of carbachol (62.5, 125 and 250 nmol/0.5 μl saline; $n = 6$ in each group) were microinjected into LH, unilaterally. The rats received carbachol once for each day during three days of conditioning phase (acquisition period). Conditioning score and

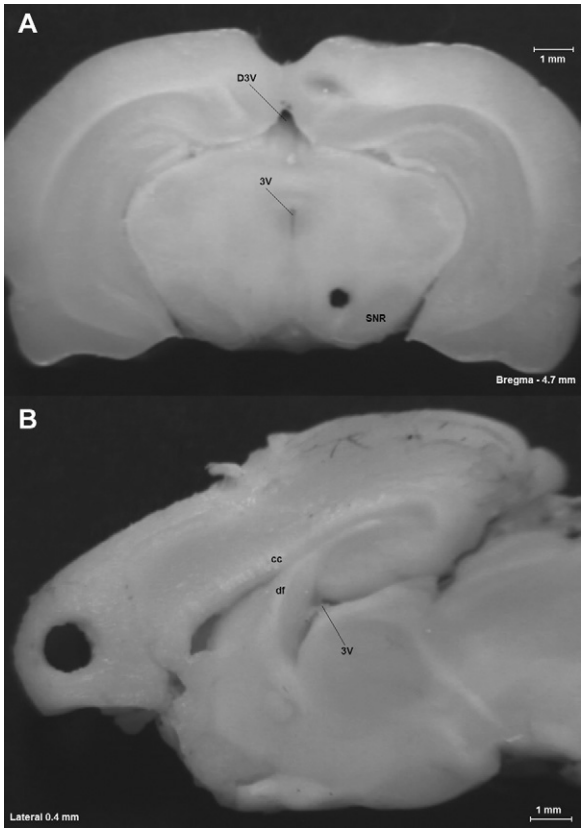

Fig. 1. A typical photomicrograph scan (250 μm) of animal's brain showing the site of (A) ventral tegmental area (coronal section), and (B) prefrontal cortex (sagittal plane) micropunch. D3V, dorsal 3rd ventricle; 3V, 3rd ventricle; SNR, substantia nigra, reticular part; cc, corpus callosum; df, dorsal fornix.

distance traveled were calculated for each rat on the test day (5th day). In control group, animals received saline (0.5 μl/rat; $n = 6$) instead of carbachol.

2.7.2. Protocol in molecular studies

For evaluation of p-CREB, p-ERK and c-fos changes in VTA, hippocampus and PFC after the acquisition of CPP induced by LH stimulation, we injected the effective dose of carbachol into LH during 3 days conditioning phase (acquisition period in CPP paradigm) in experimental groups, and in respective control groups, animals received saline as vehicle ($n = 3$ in each group). In this set of experiment, on the test day, animals were sacrificed and brains were removed. VTA, hippocampus and PFC were immediately dissected as described in Section 2.6 and delivered into liquid nitrogen. Tissues were prepared for Western blot analysis.

2.8. Statistics

In behavioral study, all data are expressed as mean ± SEM (standard error of mean). Data were analyzed by GraphPad Prism® (Version 5.0) software. In order to compare the conditioning scores and distance traveled obtained in control and experimental groups, one-way analysis of variance (ANOVA) followed by *post hoc* Dunnett's multiple comparison test was used, and for Western blot analysis, the optical densitometric data were analyzed by Mann–Whitney test and expressed as mean ± SD (standard deviation). *P*-values less than 0.05 ($P < 0.05$) were considered to be statistically significant.

3. Results

Our behavioral study showed that administration of carbachol in LH could induce CPP in a dose-dependent manner (Fig. 2A). One-way ANOVA followed by Dunnett's test [$F(3,23) = 15.33$, $P < 0.0001$] revealed that there were significant differences in conditioning scores among the vehicle (saline unilaterally microinjected into LH in a volume of 0.5 μl) and experimental group. The most effective dose of carbachol was 250 nmol/rat ($P < 0.001$). On the other hand, one-way ANOVA indicated that all different doses of carbachol did not change the locomotor activity during 10 min test period

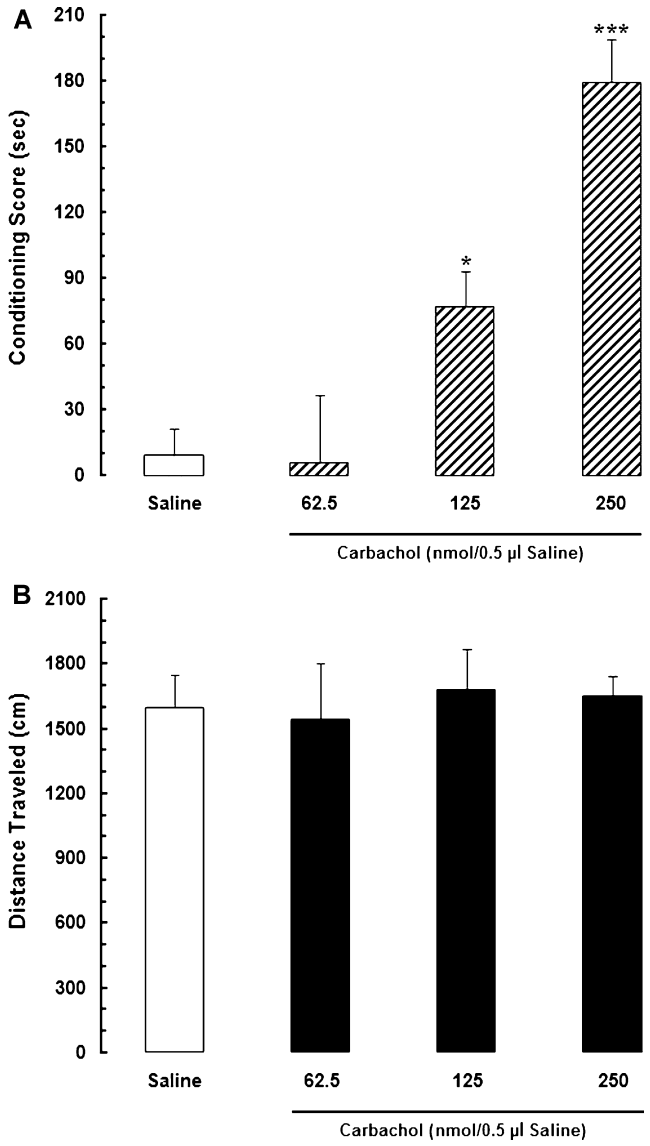

Fig. 2. Effect of unilateral administration of different doses of carbachol in lateral hypothalamus on (A) conditioning score and (B) locomotor activity (distance traveled) in rats. Dunnett's *post hoc* after ANOVA; $n = 6$ per group; mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ different from the saline control group.

(post-conditioning phase; day 5) in comparison with saline control group (Fig. 2B). In our molecular study, control and experimental animals received dose of 250 nmol/rat of carbachol during the conditioning phase for development of CPP. After conditioning, on the post-conditioning phase (5th day) instead of the test, all the rats were rapidly decapitated and VTA, hippocampus and PFC tissues were used for Western blot analysis.

3.1. Changes of CREB phosphorylation in VTA, hippocampus and PFC after the acquisition of CPP induced by LH stimulation

In this set of experiment, to investigate changes in phosphorylation of CREB in acquisition of CPP induced by intra-LH administration of carbachol, p-CREB levels were evaluated in the aforementioned regions by Western blot analysis after conditioning period. Mann–Whitney test indicated that CREB protein activation increased significantly in VTA ($P < 0.01$) and PFC ($P < 0.05$) in animals that received intra-LH carbachol during the conditioning period as compared to saline respective control groups. Densitometric analy-

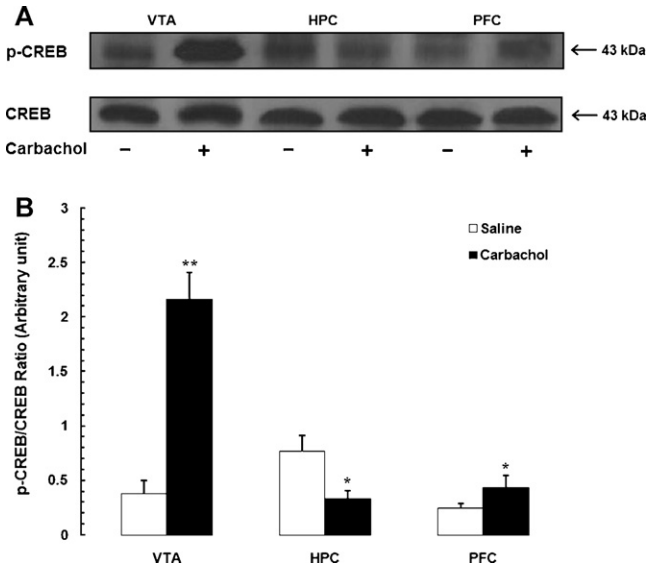

Fig. 3. Effect of LH stimulation-induced CPP on CREB phosphorylation level. (A) p-CREB level in the ventral tegmental area (VTA), hippocampus (HPC) and prefrontal cortex (PFC) was evaluated by Western blot analysis in absence (−) and presence (+) of carbachol (one representative Western blot is shown). In the absence of carbachol, animals received saline as a vehicle. (B) The densities of corresponding bands were measured and the ratio of p-CREB to CREB was calculated. Mann–Whitney test; $n = 3$ per group; mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$ different from the saline respective control group.

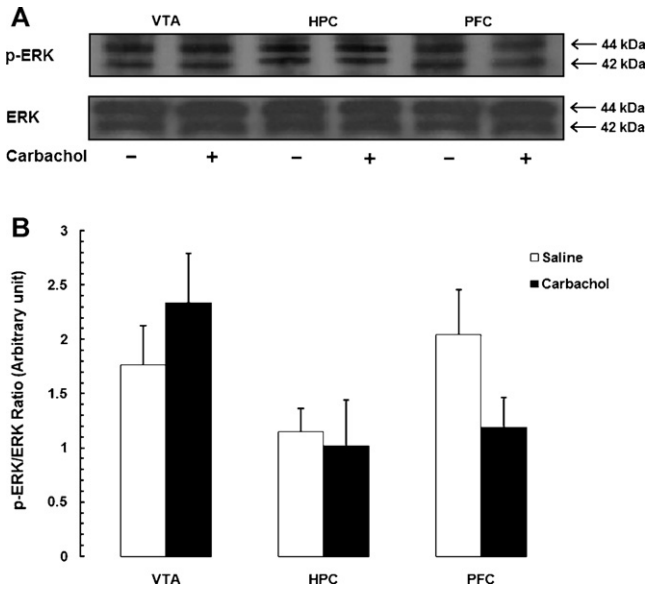

Fig. 4. ERK phosphorylation level in the rats after the acquisition of LH stimulation-induced CPP. (A) Western blot analysis was done to evaluate the p-ERK level in ventral tegmental area (VTA), hippocampus (HPC) and prefrontal cortex (PFC) in absence (−) and presence (+) of carbachol (one representative Western blot is shown). In the absence of carbachol, animals received saline as a vehicle. (B) The densities of corresponding bands were measured and the ratio of p-ERK to ERK was evaluated. Mann–Whitney test; $n = 3$ per group; mean ± SD.

sis revealed about 5-fold and 2-fold increase in p-CREB levels in VTA and PFC, respectively. However, the level of p-CREB approximately 50% decreased in hippocampus ($P < 0.05$), as shown in Fig. 3.

3.2. Changes of ERK phosphorylation in VTA, hippocampus and PFC after the acquisition of CPP induced by LH stimulation

To investigate the effect of LH stimulation-induced CPP on ERK activation in VTA, hippocampus and PFC regions, the level of phosphorylated form of ERK protein was evaluated by Western blot analysis after conditioning period in control (saline-treated) and experimental groups. Fig. 4 shows that there were no significant changes in p-ERK levels in VTA, hippocampus and PFC in animals that received intra-LH carbachol during the conditioning period as compared to saline respective control groups. However, p-ERK/ERK ratio in VTA showed enhancement, but it decreased in hippocampus and PFC.

3.3. Changes of c-fos protein levels in VTA, hippocampus and PFC after LH stimulation-induced CPP

In this set of experiment, to evaluate the changes in c-fos induction in the acquisition of CPP induced by intra-LH administration of carbachol, c-fos levels were measured in VTA, hippocampus and PFC regions by Western blot analysis after conditioning period. Obtained data in Fig. 5 revealed that after conditioning, on the CPP acquisition day, c-fos protein levels significantly increased in VTA ($P < 0.01$) and hippocampus ($P < 0.05$), while this increase in PFC was not significant in comparison with respective control groups. Our results indicated that increase in c-fos level in VTA was 12-fold, while this increase in hippocampus was 2-fold as compared to saline respective control groups.

4. Discussion

The present study indicated that administration of carbachol in LH induced CPP in a dose-dependent manner. The major findings of this study were: (1) after LH stimulation-induced CPP, level of p-CREB in VTA and PFC increased significantly; this enhancement in VTA was noticeable. But in hippocampus, p-CREB/CREB ratio decreased significantly; (2) there was no significant change in level of p-ERK after CPP acquisition in VTA, hippocampus and PFC; (3) after LH stimulation-induced CPP, c-fos level increased in these three regions, but it was not significant in PFC; this expansion in VTA was noticeable.

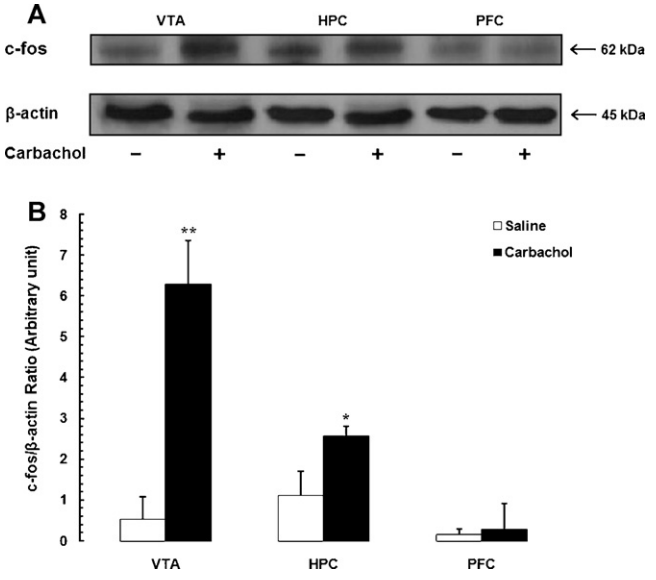

Fig. 5. Effect of LH stimulation-induced CPP on c-fos level. (A) c-fos level in ventral tegmental area (VTA), hippocampus (HPC) and prefrontal cortex (PFC) was measured by Western blot analysis in absence (−) and presence (+) of carbachol (one representative Western blot is shown). In the absence of carbachol, animals received saline as a vehicle. (B) The densities of corresponding bands were measured and their ratio to β-actin was calculated. Mann–Whitney test; $n = 3$ per group; mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$ different from the saline respective control group.

It seems that orexin-induced CPP, which is occurred after LH stimulation by carbachol, is dependent on CREB, likewise the previous studies which have shown that CREB plays an important role as a molecular basis in cannabinoid, opioid, and alcohol dependencies [31,45,46,63,69]. The noticeable enhancement of p-CREB in VTA demonstrated that this area probably plays main role in orexin-induced CPP. In agreement with our results, Brunzell et al., in 2009 showed that place conditioning-associated changes in CREB activity are necessary for nicotine CPP [8]. Also, several lines of evidence confirmed the role of place preference in changes of CREB [3,10]. Constantly, Pascual et al. revealed the activation of CREB after 3 h in CPP [46], and Brunzell et al. showed that there was an increase in NAc shell levels of p-CREB while isolated to a nicotine-paired chamber 22 h after the last period of 3 daily pairings of nicotine with that chamber [8]. It has been revealed that carbachol has a 2–5 min onset of action and its duration of action is 4–8 h [12]. Since, the elevated levels of p-CREB were observed one day after carbachol administration; we suggested that the CPP protocol and its possible impact on reward pathway may be involved in our obtained results.

There is a hypothesis that the brain's reward system includes the dopaminergic neurons originating in the midbrain [49]. VTA, the site of origin of the mesocorticolimbic DA system, is a critical site for synaptic plasticity underlying food reward-related learning [51]. It is possible that p-CREB alterations are executed within one or several particular pathways that mainly end to dopaminergic and GABAergic VTA neurons which are involved in CPP induction. In better words, some specific neurons from VTA are influenced by LH stimulation-induced CPP, that are related to LH projections to VTA, and CREB phosphorylation may increase in these neurons that needs more investigations, using immunohistochemistry and other molecular techniques. Several studies which investigated CREB levels during pain processing, also revealed that an increase in p-CREB occurs in a number of pain models [26] such as subcutaneous formalin [32,67] and neuropathic pain [4,38]. Moreover, some investigations confirmed that CREB expression enhances after stress induction [13,15]. Nevertheless, we assume that repeated exposure to LH stimulation significantly decreased level of p-CREB in hippocampus, while previous studies revealed that p-CREB in hippocampus was enhanced by morphine- [40] and nicotine- [46] induced CPP. These different results may be due to (i) the dissection of the whole hippocampus in this study. It may be better to investigate different areas of hippocampus, such as CA1, CA2 and CA3 to get more precise results. Previous studies have shown the presence of LH projection to CA1 and CA3 [62,65,66]; (ii) the orexin receptors concentration in the whole hippocampus; and (iii) the different mechanisms and intracellular signal pathways involved in orexin-induced CPP. Our observed data demonstrated that LH stimulation-induced CPP increases p-CREB in the PFC, but this alteration is lesser than that of the two other regions.

It is noticeable that there are challenging studies dealing with ERK activity within VTA. Lin et al. showed a significant increase in ERK activity in VTA following morphine-induced CPP [36]. Other investigations indicated that cocaine could enhance p-ERK in dorsal striatum [31]. In a recent study, Li et al. showed that chronic morphine treatment did not alter ERK phosphorylation in mouse NAc and frontal association cortex [35]. In agreement, obtained data in this study revealed that there was no significant alteration in the level of p-ERK in these three regions and it could be taken into consideration that ERK could not be a crucial index for LH stimulation-induced CPP. Furthermore, Fuenzalida et al. provided evidence that there are some molecular pathways in which ERK and CREB contribute, independent from each other [19].

Concurrent with recent investigations [28,29,49], c-fos changes were observed in our study. Constantly, earlier studies showed that acute morphine injection increased c-Fos protein expression in several brain areas including the dorsomedial striatum and midline/intralaminar nuclei of thalamus [20–22]. Moreover, Tolliver et al. showed that c-fos expression is essential for acquisition, but not expression of morphine-induced CPP [58]. Though, it seems that c-fos can be a good indicator, but due to increase of c-fos in all of these three areas and this fact that c-fos could be affected by different factors which implicated in some brain modulated activities such as pain [27] and stress [30,48,44], it cannot be a positive indicator for the acquisition of CPP by LH stimulation. Our results indicated the increase of c-fos level in VTA. This enhancement was also observed in hippocampus, but this alteration was lower than that of the VTA. This demonstrated the stronger interaction between LH and VTA in LH stimulation-induced CPP. Also, c-fos had no significant change in PFC. So, it could be suggested that PFC may not exert precise role in LH stimulation- or orexin-induced CPP. It is possible that PFC may be involved in rewarding response to LH stimulation via VTA, and since there are some projections from VTA to PFC, it seems that VTA plays main role in reward processing. A recent study showed that VTA exerts a complex gating action over PFC neural activity, by either facilitating or inhibiting firing in hippocampal-PFC pathway depending on the frequency and relative timing of the arrival of afferent input [18]. Although LH also has projection to hippocampus and PFC, it seems that LH projection pathway to VTA could be more important for the acquisition of CPP. Consistently, other investigations showed that LH projection to VTA is necessary for learning morphine-induced CPP [1,25].

In conclusion, our findings in this study could confirm the anatomical, behavioral and electrophysiological studies that have shown a stronger reciprocal connection between LH and VTA as compared to hippocampus and PFC regarding the reward-related behaviors. It seems that studying the intracellular signals and their changes, such as CREB, can be suitable indicators for detection of the regions which are involved in conditioned place preference induced by LH stimulation and can indicate a functional relationship between LH and other brain structures involved in reward processing in rats.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2011.01.045

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