Cellular & Molecular Biology
Cell. Mol. Biol. 2014; 60 (4): 48-55
Published online December 8, 2014 (http://www.cellmolbiol.com)
Received on October 7, 2014, Accepted on November 25, 2014.
doi : 10.14715/cmb/2014.60.4.8

Morphine-induced conditioned place preference and the alterations of p-ERK, p-CREB and c-fos levels in hypothalamus and hippocampus: The effects of physical stress

P. Pahlevani[1,2], Z. Fatahi[1], M. Moradi[3] and A. Haghparast[1]

[1] Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2] School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran
[3] Substance Abuse and Dependence Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran

Corresponding author: Abbas Haghparast, Neuroscience Research Center, Shahid Beheshti University of Medical Sciences P.O.Box 19615-1178, Tehran, Iran. E-mail: a_haghparast@sbmu.ac.ir

Abstract

The hypothalamus and hippocampus are important areas involved in stress responses and reward processing. In addition, ERK/CREB pathway plays a critical role in the control of cellular responses to stress and reward. In the current study, effects of acute and subchronic stress on the alteration of p-ERK, p-CREB and c-fos levels in the hypothalamus and hippocampus of saline- or morphine-treated animals during morphine-induced conditioned place preference (CPP) procedure were investigated. Male Wistar rats were divided into two saline- and morphine-treated supergroups. Each supergroup includes of control, acute stress and subchronic stress groups. In all of groups, the CPP procedure was done, afterward the alternation of p-ERK/ERK ratio, p-CREB/CREB ratio and c-fos level in the hypothalamus and hippocampus were estimated by Western blot analysis. The results indicated that in saline- or morphine-treated animals, p-ERK/ERK ratio, p-CREB/CREB ratio and c-fos level increased after application of acute and subchronic stress (except for p-ERK/ERK ratio in morphine-control group). Our findings revealed that in saline- or morphine-treated animals, acute and subcronic stress increased the p-ERK/ERK ratio, p-CREB/CREB ratio and c-fos level in the hypothalamus and hippocampus and this enhancement in morphine-treated animals, was more considerable than that in saline-treated animals.

Key words: Hypothalamus, Hippocampus, Reward, Forced swim stress, Conditioned place preference.

Introduction

Stressful conditions produce a series of physiological and behavioral changes that enable the individual to cope with new situations (1). It is well known that the hypothalamus (HYP) and hippocampus (HIP) are involved in neural circuits mediating response to stress and control of hypothalamic – pituitary – adrenal (HPA) axis (2, 3). In the HYP, producing neurons of corticotrophin releasing hormone (CRH) located in the paraventricular nucleus and the activity of these neurons forms the basis of the activity of the HPA axis. Different kinds of stressors regulate transcription of the CRH gene in the paraventricular nucleus (2, 4). The CRH neurons induce adrenal corticotrophic hormone (ACTH) release from the pituitary, which subsequently causes cortisol (in primates) and corticosterone (in rodents) release from the adrenal cortex (4). In addition, opiate receptors have been identified in cell bodies within the HYP (5) and it was established that this area is one of brain regions involve in the opioid addiction (6). Orexin neurons in lateral HYP play an active role in reward processing and drug of abuse. Mice with a genetic deletion of orexin completely lack conditioned place preference (CPP) for morphine (7). Moreover, previous studies reported that lesions of the ventromedial HYP in rats produced a suppression of the morphine-withdrawal syndrome (8).

In addition to the HYP, the HIP is another essential region for endocrine and behavioral responses to stress, too (9). This area is critical for learning and memory and plays an important role in the acquisition of CPP. Last studies clarified that the HIP is necessary to mediate the expression of place learning (10). Lesion or inhibition of this area disturbs acquisition and expression of cocaine-induced CPP (11). The HIP has been shown to be involved in reward-related learning, particularly when animals must remember the place and cues associated with drug administration (10).

It was reported that extracellular signal-regulated protein kinase (ERK) pathway is the major convergence point in all signal pathways, regulating cellular growth and differentiation, learning, memory formation and neuronal plasticity (12). This pathway plays a critical role in control of cellular responses to stress and rewarding effects of many drugs of abuse, such as nicotine, morphine, cocaine, and alcohol (13, 14). Two well characterized nuclear targets for ERK protein are cyclic AMP-response element binding protein (CREB) and c-fos (15). CREB has been shown to be involved in the adaptative response to drug addiction, emotional behavior and stress (16, 17). Moreover, ERK and CREB have emerged as critical points of convergence in signaling pathways regulating neuronal plasticity. When the ERK activation is depressed, the CREB-dependent plasticity will be disrupted. Similarly, CREB phosphorylation is essential for the induction of the ERK-dependent plasticity (12). Some evidence declared that c-fos is a well-known marker of cell activation (18). Various kinds of stimuli, including stressful stimuli induce c-fos expression in different regions of the brain (19). This protein contributes to the initiation and coordination of molecular cascades and cellular events required for neuroplasticity, and through this mechanism it has an established role in learning and memory (20). There-

fore, in the current study we tried to indicate the effect of acute and subchronic stress on the alterations of p-ERK/ERK ratio, p-CREB/CREB ratio and c-fos level in the hypothalamus and hippocampus of saline- or morphine-treated animals and/or to determine if change in the levels of p-ERK, p-CREB and c-fos in animals which received both stress and morphine is different from animals which received only stress or morphine.

Materials and methods

Animals

Adult male Wistar rats (Pasture Institute; Tehran, Iran) weighing 230-280 g were used in the current study. Animals were maintained under standard conditions (12 h light / 12 h dark, temperature 22 ± 2º C), food and water were given ad libitum . All investigations and procedures were conducted 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 local ethical committee, Shahid Beheshti University of Medical Sciences.

Materials

Morphine sulfate (Temed co., Tehran, Iran) was diluted in 0.9% sterile saline at a concentration of 5 mg/ml and administered subcutaneously (5 mg/kg, s.c.). Antibodies directed against ERK (Cat No. #9122), p-ERK1/2 (Thr202/Tyr204; Cat No. #4377), CREB (Cat No. #4820), p-CREB (Ser133; Cat No. # 9198), c-fos (Cat No. #2250), β-Actin (Cat No. #4967) and secondary antibody IgG (Cat No. #7074) were purchased from Cell Signaling Technology, USA. Enhanced chemiluminescence (ECL) kit was obtained from Amersham Bioscience, USA.

Conditioning apparatus and paradigm

The conditioned place preference (CPP) paradigm took place on 5 consecutive days by using an unbiased procedure. Place conditioning boxes were made of Plexiglas and consist of two sides (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 CPP paradigm. It took place in the following manner.

Pre-conditioning phase

On the first day, each rat was placed separately into the apparatus for 10 min with free access to all compartments (A, B and C), and the amount of time spent in each compartment was measured to assess unconditioned preference. (The position of the animal was defined by the position of its front paws.) In the particular experimental setup used in the present study the animals did not show a significant preference for either of the compartments (A and B) during preconditioning phase.

Conditioning phase

Place conditioning phase started 1 day after preconditioning phase for 3 days. This phase consisted of six 30-min sessions (three saline-treated and three morphine-treated). These sessions were conducted twice each day (days 2–4) with a 6-h interval. On each of these days, morphine-treated groups received one conditioning session with morphine and one with saline. During these sessions, the animals were confined to one compartment by closing the removable doors. Animals were injected with morphine or saline and immediately confined to one compartment of the apparatus. After 6-h interval, following administration of saline, the animals were confined to the other compartment. Treatment compartment and order of presentation of morphine and saline were counterbalanced for either group. In saline-treated groups, animals received saline instead of morphine. In other words, they did not receive morphine during this phase and only received saline in both sessions each day.

Post-conditioning phase

On the fifth day, the wall was removed, and each animal was allowed free access to all 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 on the pre-conditioning phase during a 10-min period (21).

Forced swim stress

The forced swim stress (FSS) was performed in a Plexiglas tank (50 cm height × 30 cm diameter) filled with 35 cm depth of water (24 ± 27 °C). Each rat was obliged to swim for 6 min once a day individually. In order to induce acute stress, the animals received FSS for 6 min just one day but, in subchronic stress groups, the animals received FSS for 6 min once a day for 3 days consecutively.

Western blot analysis

For Western blot analysis, three or four rats per group were decapitated after performance of the CPP paradigm and the brains were rapidly removed on ice. The brain was placed in a stainless steel brain matrix, and the HYP and HIP were dissected bilaterally according to the atlas of Paxinos and Watson (22). All tissues were placed into liquid nitrogen to be frozen. 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. The proteins were electrophoresed in 12% SDS–PAGE gels, transferred to poly vinylidene fluoride membranes. Blots were incubated in blocking buffer (10% nonfat dry milk powder in Tris-buffered saline containing 0.5% Tween-20, TTBS) for 1 h at room temperature (RT), were incubated with primary antibody overnight at 4° C and then washed 10 min × 3 in TTBS. Blots were incubated with secondary antibody IgG for 1 h at RT, washed 10 min × 3 in TTBS,

treated with ECL reagents and exposed to film. Data analysis was performed by Image J., measuring integrated density of bands after background subtraction. Protein concentrations were determined according to Bradford's method (23).

Experimental design

Male Wistar rats were divided into two main supergroups, saline- and morphine-treated animals. Each supergroup consists of control, acute stress and subchronic stress groups. In behavioral studies, each group consists of 6-8 animals and in molecular studies, 3-4 animals were used in each group. In all of the groups the animals passed CPP stages according CPP protocol (Electronic supplementary Fig. 1A). In saline-treated animals, control group received saline (1 ml/kg) during the conditioning phase without any stress, acute stress group received saline during the conditioning phase and also FSS on the post-conditioning phase10 min before was put on apparatus and subchronic group received FSS during 3-day schedule conditioning phase, 10 min before saline injection once a day. In morphine-treated animals, control group treated with morphine (5 mg/kg) during the conditioning phase, acute stress group received morphine in the conditioning phase, also FSS on the post conditioning phase 10 min before was put on apparatus (Electronic supplementary Fig. 1B) and group received FSS during the conditioning phase 10 min before administration of morphine (Electronic supplementary Fig. 1C).

To investigate the alterations of p-ERK/ERK ratio, p-CREB/CREB ratio and c-fos level, on the fifth day, each animal was immediately killed after post-conditioning phase by decapitation and the brains were rapidly removed (n=3-4/group). In each animal the HYP and HIP were immediately dissected out and kept at 80º C until further processed. In each area, p-ERK/ERK ratio, p-CREB/CREB ratio and c-fos level were evaluated by using Western blot analysis.

Statistical analysis

All analyses were calculated by using GraphPad Prism (Version 5.0) software. All data were shown as mean ± SEM (standard error of mean) and were analyzed by two-way or one-way ANOVA followed by *post hoc* Bonferroni test or Newman-Keuls multiple comparison test as needed, respectively. *P*-values less than 0.05 ($P<0.05$) were considered to be statistically significant.

Results

In the behavioral experiments, for determining the effects of acute and subchronic stress on the change in conditioning scores, the animals passed CPP stages according to CPP protocol and afterward, conditioning score was calculated. The behavioral data has been published in recent study (21). Briefly, the behavioral experiments showed that there are significant differences in the conditioning scores between the groups with/without stress in the saline- and morphine-treated animals [Stress effect: $F(2,35)=25.77$, $P=0.0004$; Morphine treatment: $F(1,35)=1.02$, $P=0.4413$; Stress×Morphine treatment: $F(2,35)=26.4$, $P=0.0003$]. These data also clarified that in the saline-treated animals, conditioning scores did not have any alteration in respective saline-control group after application of acute stress but it decreased after performance of the subchronic stress [$F(5,40)=8.271$, $P<0.0001$]. On the other hand, in the morphine-treated animals, conditioning scores decreased after application of both acute and subchronic stress (21).

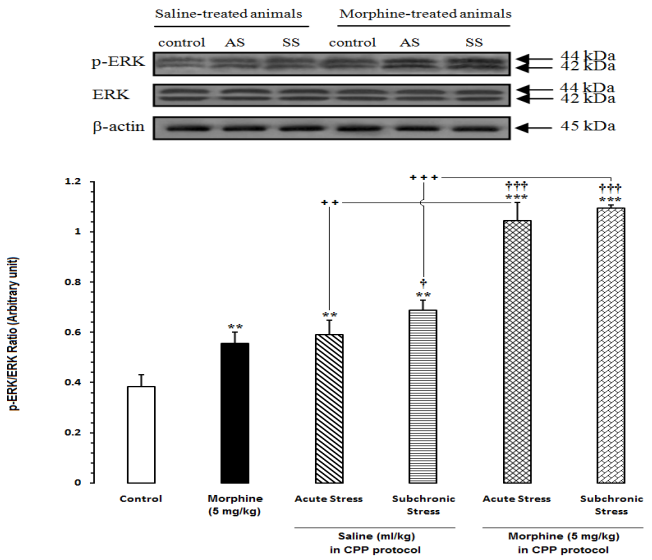

Figure 1. Alteration of p-ERK/ERK ratio in the hypothalamus (HYP) after induction of acute and subchronic stress during conditioned place preference paradigm. *Upper panels* are the representative immunoblots of proteins in this area. *Bottom panels* show the mean p-ERK/ERK ratio calculated from densitometric quantification of the corresponding bands from left to right, respectively. Each point shows the mean ± SEM for 3-4 rats.

** $P<0.01$, *** $P<0.001$ different from the saline-control group

† $P<0.05$, ††† $P<0.001$ different from the morphine-control group

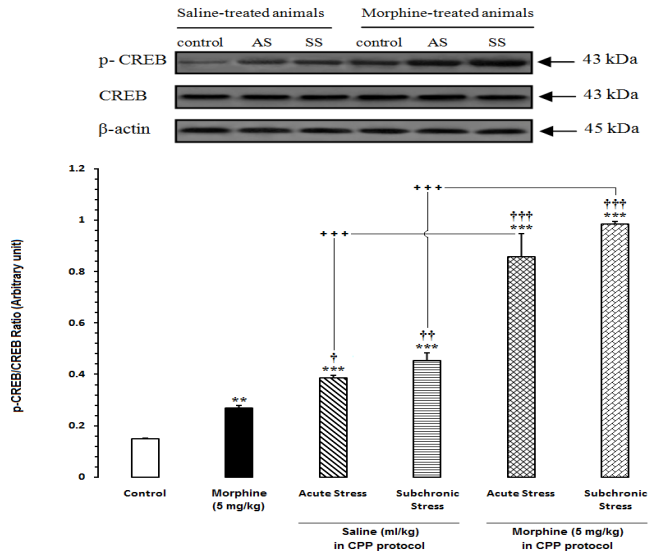

Figure 2. Alteration of p-CREB/CREB ratio in the hypothalamus (HYP) after induction of acute and subchronic stress during conditioned place preference paradigm. *Upper panels* are the representative immunoblots of proteins in this area. *Bottom panels* show the mean p-CREB/CREB ratio calculated from densitometric quantification of the corresponding bands from left to right, respectively. Each point shows the mean ± SEM for 3-4 rats.

** $P<0.01$, *** $P<0.001$ different from the saline-control group

† $P<0.05$, †† $P<0.01$, ††† $P<0.001$ different from the morphine-control group

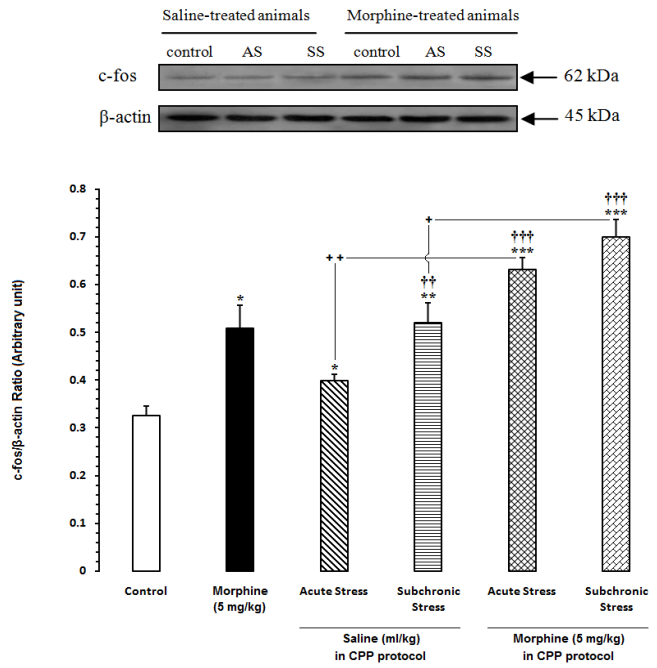

Figure 3. Alteration of c-fos in the hypothalamus (HYP) after induction of acute and subchronic stress during conditioned place preference paradigm. *Upper panels* are the representative immunoblots of proteins in this area. *Bottom panels* show the mean c-fos calculated from densitometric quantification of the corresponding bands from left to right, respectively. Each point shows the mean ± SEM for 3-4 rats.

* $P<0.05$, ** $P<0.01$, *** $P<0.001$ different from the saline-control group

†† $P<0.01$, ††† $P<0.001$ different from the morphine-control group

Changes in p-ERK/ERK ratio, p-CREB/CREB ratio and c-fos level in the hypothalamus after application of acute and subchronic stress during conditioned place preference procedure

To estimate the impress of acute and subchronic stress on the alternations of p-ERK, p-CREB and c-fos levels in the HYP of saline- or morphine-treated animals, the CPP paradigm was done and afterward the changes in p-ERK/ERK ratio, p-CREB/CREB ratio and c-fos level were measured by Western blot analysis. Two-way ANOVA followed by Bonferroni test for p-ERK/ERK ratio [Stress effect: $F(2,12)=240.5$, $P<0.0001$; Morphine treatment: $F(1,12)=21.61$, $P=0.0006$; Stress×Morphine treatment: $F(2,12)=2.34$, $P=0.1386$; Fig. 1], p-CREB/CREB ratio [Stress effect: $F(2,12)=519.3$, $P<0.0001$; Morphine treatment: $F(1,12)=31.41$, $P=0.0002$; Stress×Morphine treatment: $F(2,12)=1.06$, $P=0.3768$; Fig. 2] and c-fos [Stress effect: $F(2,12)=137.8$, $P<0.0001$; Morphine treatment: $F(1,12)=89.59$, $P<0.0001$; Stress×Morphine treatment: $F(2,12)=6.592$, $P=0.0117$; Fig. 3] showed that all measured factors were increased significantly in saline- or morphine-treated animals after application of acute and subchronic stress in comparison with saline- or morphine-control groups, respectively.

Changes in p-ERK/ERK ratio, p-CREB/CREB ratio and c-fos level in the hippocampus after application of acute and subchronic stress during conditioned place preference procedure

We examined the effects of acute and subchronic stress on the variation of p-ERK, p-CREB and c-fos levels in the HIP by using Western blot analysis after performance of the CPP procedure. Two-way ANOVA followed by Bonferroni test for p-ERK/ERK ratio [Stress effect: $F(2,12)=41.45$, $P<0.0001$; Morphine treatment:

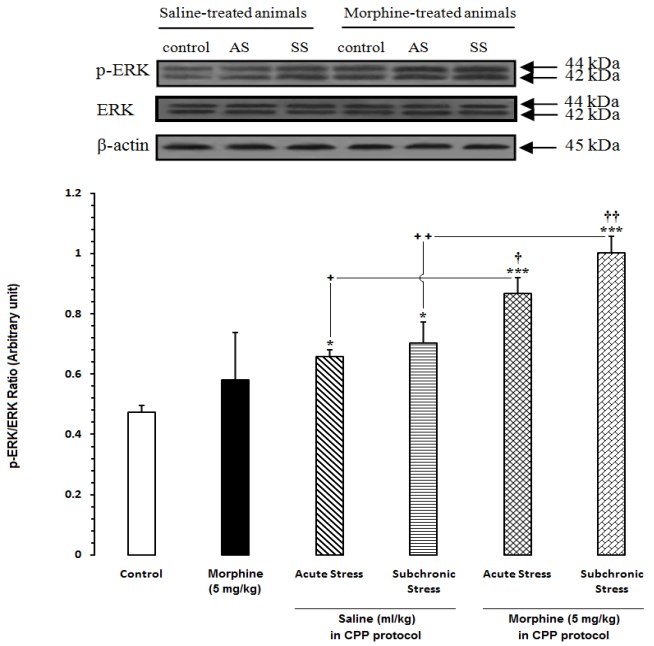

Figure 4. Alteration of p-ERK/ERK ratio in the hippocampus (HIP) after induction of acute and subchronic stress during conditioned place preference paradigm. *Upper panels* are the representative immunoblots of proteins in this area. *Bottom panels* show the mean p-ERK/ERK ratio calculated from densitometric quantification of the corresponding bands from left to right, respectively. Each point shows the mean ± SEM for 3-4 rats.

* $P<0.05$, *** $P<0.001$ different from the saline-control group

† $P<0.05$, †† $P<0.01$ different from the morphine-control group

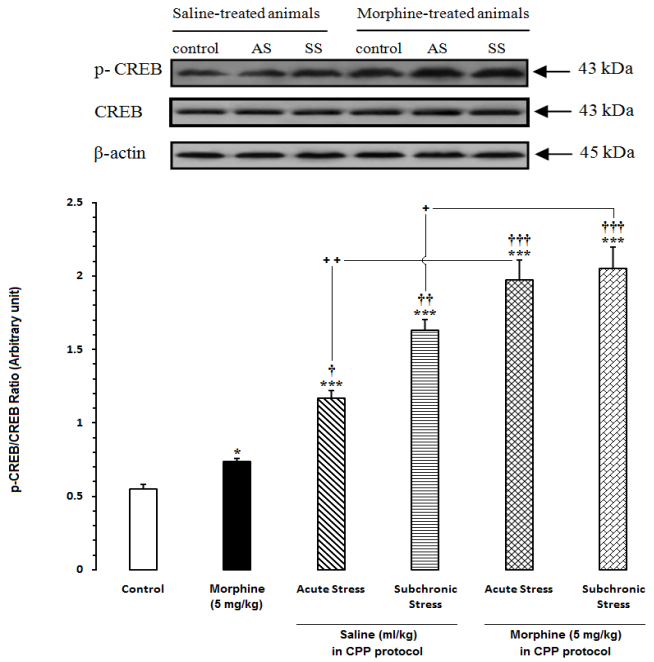

Figure 5. Alteration of p-CREB/CREB ratio in the hippocampus (HIP) after induction of acute and subchronic stress during conditioned place preference paradigm. *Upper panels* are the representative immunoblots of proteins in this area. *Bottom panels* show the mean p-CREB/CREB ratio calculated from densitometric quantification of the corresponding bands from left to right, respectively. Each point shows the mean ± SEM for 3-4 rats.

* $P<0.05$, *** $P<0.001$ different from the saline-control group

† $P<0.05$, †† $P<0.01$, ††† $P<0.001$ different from the morphine-control group

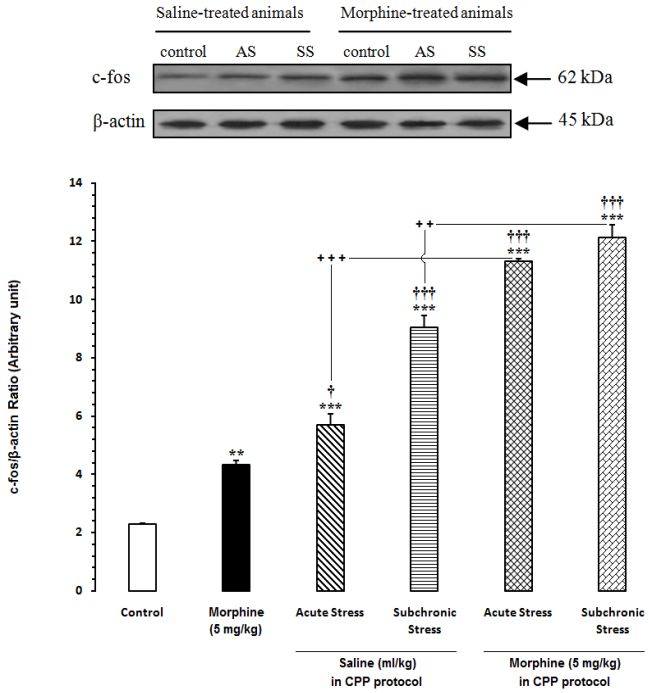

Figure 6. Alteration of c-fos in the hippocampus (HIP) after induction of acute and subchronic stress during conditioned place preference paradigm. *Upper panels* are the representative immunoblots of proteins in this area. *Bottom panels* show the mean c-fos calculated from densitometric quantification of the corresponding bands from left to right, respectively. Each point shows the mean ± SEM for 3-4 rats.

** $P<0.01$, *** $P<0.001$ different from the saline-control group

† $P<0.05$, ††† $P<0.001$ different from the morphine-control group

F(1,12)=6.697, P=0.0237; Stress×Morphine treatment: F(2,12)=0.4764, P=0.6323; Fig. 4], p-CREB/CREB ratio [Stress effect: F(2,12)=341.8, P<0.0001; Morphine treatment: F(1,12)=31.97, P=0.0001; Stress×Morphine treatment: F(2,12)=7.196, P=0.0088; Fig. 5] and c-fos [Stress effect: F(2,12)=1230, P<0.0001; Morphine treatment: F(1,12)=221.5, P<0.0001; Stress×Morphine treatment: F(2,12)=28.06, P<0.0001; Fig. 6] revealed that in saline- or morphine-treated animals p-ERK/ERK ratio, p-CREB/CREB ratio and c-fos level were enhanced significantly. However, the increase of p-ERK/ERK ratio in morphine-control group was not significant as compare with saline-control group.

Discussion

The major finding in this study showed that acute and subchronic stress enhanced the levels of p-ERK, p-CREB and c-fos in the hypothalamus and hippocampus of saline- or morphine-treated animals, also this increase in morphine-treated animals was more noteworthy than saline-treated animals. In this study, to induce stress we used forced swim stress because it has been shown that forced swimming leads to adrenocorticotrophic hormone and corticosterone release. Both hormones increased significantly up to 40 min after stress (24, 25, 26). In saline- or morphine- treated animals, p-ERK/ERK ratio, p-CREB/CREB ratio and c-fos level increased in compare with saline- or morphine-control groups, respectively. However, the increscent of p-ERK/ERK ratio in morphine-control group was not significant as compare with saline-control group in the HIP. Several studies pointed that ERK activation occurs in response to a variety of extracellular stimuli and forms an essential pathway for cells to generate adaptive responses to changing environments (27). ERK phosphorylation triggers downstream activation of the transcription factors CREB and enhanced the level of c-fos protein (28, 29). Furthermore, assessment of c-fos level is a widely used method to label neuronal activation in the brain (30) because this protein is considered to reflect neuronal activities due to its function in controlling gene transcription via binds to regulatory sites on DNA (31). Substantial evidence indicated that ERK pathway mediated the basic mechanism of morphine reward in the nucleus accumbens, prefrontal cortex and HIP of rats (17) and changes in the expression or activity of ERK and CREB are involved in the development of behavioral sensitization and increased drug-taking behavior (32). Besides, it was demonstrated that ERK pathway participated in stress response and coped with stressful stimuli (12, 19). In this study all cited factors were increased in both regions except for p-ERK/ERK ratio in morphine-control group in the HIP, but as regards in this region downstream targets of p-ERK such as p-CREB and c-fos were enhancement significantly, it could suggest that the increase p-ERK/ERK ratio was significant but it was returning to basal level when we dissected out the tissue.

In saline-treated animals, application of acute and subchronic stress induced the increase of p-ERK, p-CREB and c-fos levels in both regions. It is noticeable that there are challenging studies about effects of stress on ERK/CREB pathway activation and both increase and decrease of this pathway activation have been reported. In agreement with our result, several studies have shown that different kinds of stressors activated ERK/CREB pathway in neural cells. Social defeat stress increased c-fos expression in mesocorticolimbic terminal regions such as the nucleus accumbens, amygdala and prefrontal cortex, which persists up to 14 days after stress termination (33), it also enhanced p-CREB in the periaquaductal gray (34) and hyperactivation of ERK in the HIP and prefrontal cortex (35). Forced swim stress caused a significant increase in phosphorylation of ERK and CREB in the nucleus accumbens, amygdala, striatum and dentate gyrus of the HIP (34, 36). Besides, footshock stress led to an elevation in p-CREB in the amygdala, parietal cortex, paraventricular nucleus of the HYP and HIP (34). In contrast, Grønli et al. (2006) showed that chronic mild stress resulted in a significant decrease in CREB activation in the dentate gyrus but not in the cornus ammonis region as well as Musazzi et al. (2010) reported that chronic corticosterone administration increased depressive-like behavior and reduced phosphorylation of ERK in dentate gyrus (35, 37). Moreover, it was shown that sustained exposure to stressful conditions, in experimental animals, was linked with elevated glucocorticoid levels and reduced CREB expression in the HIP (38). Therefore, it could be concluded that the effects of stress on the change in the activation of ERK/CREB pathway depends on several factors such as stress protocol, stress induction period and brain areas studied, also application of acute or subchronic swim stress causes the activation of this pathway in the rat HYP and HIP.

In other part of our study, we evaluated the effects of acute and subchronic stress on the change in the activation of ERK/CREB pathway in morphine-treated animals. Our result revealed that p-ERK/ERK ratio, p-CREB/CREB ratio and c-fos level increased significantly in both areas. Notably, these factors enhanced significantly in morphine-control group in comparison with saline-control group. Previous studies showed that nicotine self-adminstration reduced CREB phosphorylation in the nucleus accumbens (32) and receiving chronic nicotine in drinking water resulted in the decrease of CREB phosphorylation in the nucleus accumbens and the increase of it in the prefrontal cortex (39). Additionally, acute nicotine administration subcutaneously increased c-fos mRNA expression in the bed nucleus of the stria terminalis, nucleus accumbens and ventral tegmental area in adolescent rats (40, 41). Other investigations indicated that cocaine could enhance p-ERK in dorsal striatum but did not alter the level of p-CREB in this region (15). Mice lacking of CREB did not respond to a low dose of morphine that is rewarding in wild type mice in a conditioned place preference paradigm (42). Moreover, acute cocaine injections rapidly (within 5– 20 min) increased ERK phosphorylation in the dorsal striatum and nucleus accumbens (28). It was also observed that CREB and p-CREB protein levels were significantly decreased in the medial and basolateral, but not in the central amygdala during nicotine withdrawal (18 h) after chronic nicotine exposure (43). Our result showed that the injection of morphine (5 mg/kg, s.c.) induced the increase of p-ERK, p-CREB and c-fos levels in the HYP and HIP. Therefore, it seems that the effect of opioids on the change in ERK/CREB pathway activation relates to various items including the duration of drug receiving, route of administration and dosage of the drug. Interestingly, the effect of acute and subchronic stress on the activation of ERK/CREB pathway in morphine-treated animals was more considerable than that of saline-treated animals which represented morphine amplified the effects of stress on the activation of this pathway. As mentioned above, forced swim stress leads to the increase of corticosterone. In addition, our results indicated that exposure animals to forced swim stress increased p-ERK, p-CREB and c-fos levels. So, it seems that exposure to forced swim stress led to corticosterone release which, in turn, caused to the increase of p-ERK, p-CREB and c-fos levels. On the other hand, previous studies and also our result showed that morphine increased mentioned factors in neural cells. Therefore, it could mention that in morphine-treated animals, concurrent exposure to morphine and stress resulted in more enhancements of these factors than saline-treated animals. Besides, behavioral data showed that conditioning scores decreased in the saline-treated animals after the application of subchronic stress and in the morphine-treated animals after application both acute and subchronic stress. Last studies clarified that ERK/CREB pathway has an important role in the memory formation and adaptive responses to environmental changes (27). Therefore, it could be suggested that the activation of ERK/CREB pathway leads to memory formation and finally aversion to the compartment which the animal was placed in after getting stress. Nevertheless, there is evidence that showed chronic administration of heroin hampered mice cognitive abilities in the Morris water maze, a test which is considered a very useful tool to evaluate rodent spatial learning and memory capabilities (44) and rat offspring from morphine-addicted mothers showed impaired performance in spatial working and cued reference memory (45). However, duration of drug administration and used doses of drugs in these studies were different form our study. On the other hand, previous studies revealed that administration of morphine (5 mg/kg, s.c.) in CPP protocol led to preference to place which animals received morphine there (46). So, it seems that observed aversion in this study did not relate to impair memory by morphine and it likely related to ERK/CREB pathway activation. However, further studies are needed to confirm this. This finding can help to understand effects of stress or addiction on the cellular signaling pathways and may be helpful (accompany with future supplementary studies) to find a new clinical approach for treatment of stress or addiction. In conclusion, acute and subchronic stress increase the activation of ERK/CREB pathway in the hypothalamus and hippocampus of saline- and/or morphine-treated animals, meanwhile morphine amplifies the effect of stress on the enhancement of this pathway.

Acknowledgements

This work was supported by the grant (No. 91003540) from Iran National Science Foundation, Tehran, Iran.

References

1. Da Silva Torres, I.L., Cucco, S.N., Bassani, M., Duarte, M.S., Silveira, P,P., Vasconcellos, A.P., Tabajara, A.S., Dantas, G., Fontella, F.U., Dalmaz, C. and Ferreira, M.B., Long-lasting delayed hyperalgesia after chronic restraint stress in rats-effect of morphine administration. *Neurosci. Res.* 2003, **45**: 277-283. doi:10.1016/S0168-0102(02)00232-8.

2. Givalois, L., Arancibia, S. and Tapia-Arancibia, L., Concomitant changes in CRH mRNA levels in rat hippocampus and hypothalamus following immobilization stress. *Mol. Brain Res.* 2000, **75**: 166-171. doi:10.1016/S0169-328X(99)00290-9.

3. Rodrigues, Siqueira, I., Fochesatto, C., da Silva Torres, I.L., Dalmaz, C. and Alexandre Netto, C., Aging affects oxidative state in hippocampus, hypothalamus and adrenal gland of Wistar rats. *Life Sci.* 2005, **78**: 271-278. doi:10.1016/j.lfs.2005.04.044.

4. Bao, A.M., Meynen, G. and Swaab, D.F., The stress system in depression and neurodegeneration: Focus on the human hypothalamus. *Brain Res. Rev.* 2008, **57**: 531-553. doi:10.1016/j.brainresrev.2007.04.005.

5. Ueyama, T., Tanioku, T., Nuta, J., Kujira, K., Ito, T., Nakai, S. and Tsuruo, Y., Estrogen alters c-Fos response to immobilization stress in the brain of ovariectomized rats. *Brain Res*. 2006, **1084**: 67-79. doi:10.1016/j.brainres.2006.02.008.

6. Guo, Y., Wang, H.L., Xiang, X.H. and Zhao, Y., The role of glutamate and its receptors in mesocorticolimbic dopaminergic regions in opioid addiction. *Neurosci. Biobehav. Rev.* 2009, **33**: 864-873. doi:10.1016/j.neubiorev.2009.02.005.

7. Aston-Jones, G., Smith, R.J., Sartor, G.C., Moorman, D.E., Massi, L., Tahsili-Fahadan, P. and Richardson, K.A., Lateral hypothalamic orexin/hypocretin neurons: A role in reward-seeking and addiction. *Brain Res.* 2010, **1314**: 74-90. doi:10.1016/j.brainres.2009.09.106.

8. Prieto-Gomez, B., Reyes-Vazquez, C. and Dafny, N., Microiontophoretic application of morphine and naloxone to neurons in hypothalamus of rat. *Neuropharmacology* 1984, **23**: 1081-1089.

doi:10.1016/0028-3908(84)90133-3.
9. Hays, S.L., McPherson, R.J., Juul, S.E., Wallace, G., Schindler, A.G., Chavkin, C. and Gleason, C.A., Long-term effects of neonatal stress on adult conditioned place preference (CPP) and hippocampal neurogenesis. *Behav. Brain Res.* 2012, **227**: 7-11. doi:10.1016/j.bbr.2011.10.033.
10. Rezayof, A., Zatali, H., Haeri-Rohani, A. and Zarrindast, M.R., Dorsal hippocampal muscarinic and nicotinic receptors are involved in mediating morphine reward. *Behav. Brain Res.* 2006, **166**: 281-290. doi:10.1016/j.bbr.2005.08.010.
11. Cui, Y., Zhang, X.Q., Cui, Y., Xin, W.J., Jing, J. and Liu, X.G., Activation of phosphatidylinositol 3-kinase/Akt-mammalian target of Rapamycin signaling pathway in the hippocampus is essential for the acquisition of morphine-induced place preference in rats. *Neuroscience* 2010, **171**: 134-143. doi:10.1016/j.neuroscience.2010.08.064.
12. Qi, X., Lin, W., Li, J., Li, H., Wang, W., Wang, D. and Sun, M., Fluoxetine increases the activity of the ERK-CREB signal system and alleviates the depressive-like behavior in rats exposed to chronic forced swim stress. *Neurobiol, Dis,* 2008, **31**: 278-285. doi:10.1016/j.nbd.2008.05.003.
13. Haghparast, A., Taslimi, Z., Ramin, M., Azizi, P., Khodagholi, F. and Hassanpour-Ezatti, M., 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. *Behav. Brain Res.* 2011, **220**: 112-118. doi:10.1016/j.bbr.2011.01.045.
14. Liu, Y., Wang, Y., Jiang, Z., Wan, C., Zhou, W. and Wang, Z., The extracellular signal-regulated kinase signaling pathway is involved in the modulation of morphine-induced reward by mPer1. *Neuroscience* 2007, **146**: 265–271. doi:10.1016/j.neuroscience.2007.01.009.
15. Jenab, S., Festa, E.D., Nazarian, A., Wu, H.B., Sun, W.L., Hazim, R., Russo, S.J. and Quinones-Jenab, V., Cocaine induction of ERK proteins in dorsal striatum of Fischer rats. *Mol. Brain Res.* 2005, **142**: 134-138. doi:10.1016/j.molbrainres.2005.08.015.
16. Alboni, S., Tascedda, F., Corsini, D., Benatti, C., Caggia, F., Capone, G., Barden, N., Blom, JM. and Brunello, N., Stress induces altered CRE/CR EB pathway activity and BDNF expression in the hippo campus of glucocorticoid receptor-impaired mice. *Neuropharmacology* 2011, **60**: 1337-1346. doi:10.1016/j.neuropharm.2011.01.050.
17. Wan, L., Su, L., Xie, Y., Liu, Y., Wang, Y. and Wang, Z., Protein receptor for activated C kinase 1 is involved in morphine reward in mice. *Neuroscience* 2009, **161**: 734-742. doi:10.1016/j.neuroscience.2009.03.064.
18. Igelstrom, K.M., Herbison, A.E. and Hyland, B.I., Enhanced c-Fos expression in superior colliculus, paraventricular thalamus and septum during learning of cue-reward association. *Neuroscience* 2010, **168**: 706-714. doi:10.1016/j.neuroscience.2010.04.018.
19. Senba, E., Matsunaga, K., Tohyama, M. and Noguchi, K., Stress-induced c-fos expression in the rat brain: activation mechanism of sympathetic pathway. *Brain Res. Bull.* 1993, **31**: 329-344. doi:10.1016/0361-9230(93)90225-Z.
20. Johnson, Z.V., Revis, A.A., Burdick, M.A. and Rhodes, J.S., A similar pattern of neuronal Fos activation in 10 brain regions following exposure to reward- or aversion-associated contextual cues in mic. *Physiol. Behav.* 2010, **99**: 412-418. doi:10.1016/j.physbeh.2009.12.013.
21. Haghparast, A., Fatahi, Z., Zeighamy Alamdary, S. and Khodagholi, F., Changes in apoptotic factors in hypothalamus and hippocampus after acute and subchronic stress induction during conditioned place preference paradigm. *EXCLI J.* 2013, **12**: 1001-1016.
22. Paxinos, G. and Watson, C., The rat brain in stereotaxic coordinates. 2007, 6th edition, *Elsevier*, San Diego.
23. Bradford, M., A rapid and sensitive method for the quantitation of micro-gram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 1976, **72**: 248-254.
24. Abel, E.L., Behavioral and physiological effects of different water depths in the forced swim test. *Physil. Behav*. 1994, **56**: 411-414. doi:10.1016/0031-9384(94)90215-1.
25. Rittenhouse, P.A., Lopez-Rubalcava, C., Stanwood, G.D. and Lucki, I., Amplified behavioral and endocrine responses to forced swim stress in the Wistar-Kyoto rat. *Psychoneuroendocrinology* 2002, **27**: 303-318. doi:10.1016/S0306-4530(01)00052-X.
26. Shishkina, G.T., Kalinina, T.S., Berezova, I.V., Bulygina, V.V. and Dygalo, N.N. Resistance to the development of stress-induced behavioral despair in the forced swim test associated with elevated hippocampal Bcl-xl expression. *Behav. Brain Res.* 2010, **213**: 218-224. doi:10.1016/j.bbr.2010.05.003.
27. Shiflett, M.W. and Balleine, B.W., Contributions of ERK signaling in the striatum to instrumental learning and performance. *Behav. Brain Res*. 2011, **218**: 240-247. doi:10.1016/j.bbr.2010.12.010.
28. Lu, L., Koya, E., Zhai, H., Hope, B.T. and Shaham, Y., Role of ERK in cocaine addiction. *Tren. Neurosci*. 2006, **29**: 695-703. doi:10.1016/j.tins.2006.10.005.
29. Shaul, Y.D. and Seger, R., The MEK/ERK cascade: From signaling specificity to diverse functions. *Biochim. Biophys. Acta.* 2007, **1773**: 1213-1226. doi:10.1016/j.bbamcr.2006.10.005.
30. Kuipers, S.D., Trentani, A., Westenbroek, C., Bramham, CR., Korf, J., Kema, I.P., Ter Horst, G.J. and Den Boer, J.A., Unique patterns of FOS, phospho-CREB and BrdU immunoreactivity in the female rat brain following chronic stress and citalopram treatment. *Neuropharmacology* 2006, **50**: 428-240. doi:10.1016/j.neuropharm.2005.10.006.
31. Nathaniel, T.I., Panksepp, J. and Huber, R., Alteration of c-Fos mRNA in the accessory lobe of crayfish is associated with a conditioned-cocaine induced reward. *Neurosci. Res.* 2012, **72**: 243-256. doi:10.1016/j.neures.2011.11.009.
32. Leao, R.M., Cruz, F.C., Marin, M.T. and Planeta Cda, S., Stress induces behavioral sensitization, increases nicotine-seeking behavior and leads to a decrease of CREB in the nucleus accumbens. *Pharmacol. Biochem. Behav.* 2012, **101**: 434-442. doi:10.1016/j.pbb.2012.01.025.
33. Nikulina, E.M., Arrillaga-Romany, I., Miczek, K.A. and Hammer, Jr. RP. Long-lasting alteration in mesocorticolimbic structures after repeated social defeat stress in rats: time course of mu-opioid receptor mRNA and FosB/DeltaFosB immunoreactivity. *Eur. J. Neurosci*. 2008, **27**: 2272-2284. doi:10.1111/j.1460-9568.2008.06176.x.
34. Briand, L.A. and Blendy, J.A., Molecular and genetic substrates linking stress and addiction. *Brain Res.* 2010, **1314**: 219-234. doi:10.1016/j.brainres.2009.11.002.
35. Musazzi, L., Mallei, A., Tardito, D., Gruber, S.H., El Khoury, A., Racagni, G., Mathé, A.A. and Popoli, M., Early-life stress and antidepressant treatment involve synaptic signaling and Erk kinases in a gene-environment model of depression. *J. Psychiatr. Res.* 2010, **44**: 511-520. doi:10.1016/j.jpsychires.2009.11.008.
36. Bruchas, M.R., Xu, M. and Chavkin, C., Repeated swim stress induces kappa opioid-mediated activation of extracellular signal-regulated kinase 1/2. *Neuroreport* 2008, **19**: 1417-1422. doi:10.1097/WNR.0b013e32830dd655.
37. Grønli, J., Bramham, C., Murison, R., Kanhema, T., Fiske, E., Bjorvatn, B., Ursin, R. and Portas, C.M., Chronic mild stress inhibits BDNF protein expression and CREB activation in the dentate gyrus but not in the hippocampus proper. *Pharmacol. Biochem. Behav*. 2006, **85**: 842-849. doi:10.1016/j.pbb.2006.11.021.
39. Brunzell, D.H., Russell, D.S. and Picciotto, M.R., In vivo nicotine treatment regulates mesocorticolimbic CREB and E RK

signaling in C57B l/6J mice. *J. Neurochem.* 2003, **84**: 1431-1441. doi:10.1046/j.1471-4159.2003.01640.x.

40. Kiba, H. and Jayaraman, A., Nicotine induced c-los expression in the striatum is mediated mostly by dopamine D 1 receptor and is dependent on NMDA stimulation. *Mol. Brain Res.* 1994, **23**: 1-13.

41. Shram, M.J., Funk, D., Li, Z. and Le, A.D., Acute nicotine enhances c-fos mRNA expression differentially in reward-related substrates of adolescent and adult rat brain. *Neurosci*. *Lett*. 2007, **418**: 286-291. doi:10.1016/j.neulet.2007.03.034.

42. Walters, CL. and Blendy, J,A., Different requirements for cAMP response element binding protein in positive and negative reinforcing properties of drugs of abuse. *J. Neurosci.* 2001, **21**: 9438-9444.

43. Pandey, S.C., Roy, A., Xu, T. and Mittal, N., Effects of protracted nicotine exposure and withdrawal on the expression and phosphorylation of the CREB gene transcription factor in rat brain. *J. Neurochem.* 2001, **77**: 943-952. doi:10.1046/j.1471-4159.2001.00309.x.

44. Tramullas, M., Martinez-Cue, C. and Hurle, M.A., Chronic administration of heroin to mice produces up-regulation of brain apoptosis-related proteins and impairs spatial learning and memory. *Neuropharmacology* 2008, **54**: 640-652. doi:10.1016/j.neuropharm.2007.11.018.

45. Lin, C.S., Tao, P.L., Jong, Y.J., Chen, W.F., Yang, C.H., Huang, L.T., Chao, C.F. and Yang, SN., Prenatal morphine alters the synaptic complex of post synapyic density 95 with N-methyl aspartate receptor subunit in hippocampal CA1 subregion of rat offspring leading to long-term cognitive deficits. *Neuroscience* 2009, **158**: 1326-1337. doi:10.1016/j.neuroscience.2008.11.007

46. Zarrindast, M.R., Fattahi, Z., Rostami, P. and Rezayof, A., Role of the cholinergic system in the rat basolateral amygdala on morphine-induced conditioned place preference. *Pharmacol. Biochem. Behav.* 2005, **82**: 1-10. doi:10.1016/j.pbb.2005.02.018.