Original Article

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Investigating the Role of P-CREB and c-Fos protein Expression in Nicotine-Induced Anxiogenesis in Rats

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ABSTRACT

BACKGROUND AND OBJECTIVE: Nicotine is one of the most common addictive substances that has many effects on the central nervous system, including dose-dependent anxiety that is mediated by various proteins. Since the mechanisms and proteins involved in nicotine anxiety are unclear, the purpose of the present study was to investigate the role of p-CREB (cAMP Response Element-Binding Protein) and c-Fos proteins in the basolateral amygdala (BLA) on nicotine-induced anxiety behaviours.

METHODS: In this experimental study, 24 male Wistar rats were divided into three groups of 7 (saline, nicotine 0.3 and 0.7 mg/kg, intraperitoneally) in behavioral experiment and four groups of 3 (control: no injection and test, Saline, nicotine 0.3 and 0.7 mg/kg) in immunohistochemical experiments. Anxiety-like behaviours were evaluated with % OAT (Open Arm Time), %OAE (Open Arm Entry), and locomotor activity in the elevated plus maze. The expression of p-CREB and c-Fos proteins in BLA region was also assessed by immunohistochemistry.

FINDINGS: Intraperitoneal administration of nicotine at a dose of 0.7mg/kg decreased %OAT (5.4±0.42) and %OAE (29.4±0.61) compared to saline group (15.2±0.82) and (42.1±0.45), indicating an anxiety-like effect (respectively, $p<0.001$, $p<0.01$). In addition, there was a significant difference in the expression of p-CREB ($H(3)= 6.99$, $p<0.05$) and c-Fos ($H(3)=13.11$, $p<0.01$) protein in the BLA between treatment groups. P-CREB protein expression was higher in the BLA area of control group than in the other groups. C-Fos protein expression was significantly lower in the BLA region of the animals of control, nicotine 0.3 mg/kg and 0.7 mg/kg groups compared to saline group ($p<0.01$).

CONCLUSION: The results of this study indicated that systemic administration of nicotine induced anxiety-like behaviors at high doses. Also, the expression of P-CREB and c-Fos protein was unchanged and decreased in the treatment groups, respectively.

KEY WORDS: *Nicotine, Anxiety, Basolateral amygdala, Immunohistochemistry.*

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Introduction

Drug abuse has increased dramatically in recent years. Nicotine is one of the main addictive substances obtained through cigarette smoke and has numerous effects on various body systems including the central nervous system. One of these complications is nicotine-induced dose-dependent anxiety (1). From a physiological perspective, anxiety is a complex reaction that results from various biochemical and endocrine consequences (2). The most important of these can be a significant increase in the release of norepinephrine in various brain regions including the amygdala (3). The amygdala is one of the major components of the limbic system and plays an important role in the neurobiology of anxiety and is associated with many emotional centers in the brain (4).

Different neurotransmitter systems in the amygdala modulate anxiety-like behaviors (5). Among these neurotransmitters, norepinephrine plays an important role in modulating anxiety in animal models (6). Norepinephrine binds to adrenergic receptors and initiates a cascade of intracellular events by which phosphorylation (cAMP Response Element-Binding Protein) occurs, phosphorylated CREB by binding to its specific site increases transcription of some genes involved in the process of anxiety, including the fos gene, and increases c-Fos expression in various areas of the brain, including the basolateral amygdala (BLA), which play an important role in anxiety (7,8).

Studies have shown that systemic administration of nicotine in specific amounts increases anxiety in various types of behavioral models such as Elevated Plus Maze (EPM). It has also been shown that nicotine induces anxiogenic effects through interaction with many neurotransmitter systems at different brain sites (9). Despite various studies, there is little information about the mechanisms of nicotine function on amygdala neuronal activity in vivo. In addition, the pathways through which nicotine exerts anxiogenic and anxiolytic effects have not been well characterized.

Conducting basic studies in this area and increasing awareness of the mechanisms of action in the brain can help our understanding of the cause and treatment of anxiety disorders and pave the way for future studies to reduce the harmful effects of smoking on anxiety. Understanding the action of the molecular cascades involved after nicotine treatment is one of the most important factors in understanding the pathways of anxiety and can be used with effective drugs to treat the adverse effects of their use in treatment of addiction. Therefore, the aim of the present study was to investigate the role of c-Fos and p-CREB proteins in the BLA region on nicotine-induced anxiety-like behaviors using EPM.

Methods

This experimental study was approved by the Ethics Committee of Tehran University of Medical Sciences under the code of ethics IR. TUMS. REC. 2013. 51. Twenty-four Wistar male rats weighing 200-250 g were provided and kept in standard cages at standard temperature (22±2° C) and light (12-hour dark and light cycles) with adequate access to water and food. Animals were moved by hand five days before the experiments and twice daily for five minutes to reduce stress. All tests were conducted from nine in the morning until three in the afternoon. Three groups of animals (saline, nicotine 0.3 and 0.7 mg/kg intraperitoneally) were used for behavioral tests, each of which contained 7 animals and each animal was used once for testing. Four groups of three animals (control (without injections and tests), saline, nicotine 0.3 and 0.7 mg/kg) were used.

Drugs: Nicotine (Sigma, USA) was dissolved in saline and adjusted to a pH of 7.2 to 7.4 using a sodium hydroxide solution one tenth and one hundredth normalities. The drug was injected intraperitoneally at 1 ml/kg and at doses of 0.3 and 0.7 mg/kg. Saline was injected as a control group at a rate of 1 ml/kg. Dose selection was based on previous research (10).

EPM: There are several ways to measure anxiety in laboratory animals (11), including EPM as one of the best methods to evaluate the effects of substances on anxiety in laboratory animals. The EPM is made of wood and has four arms in the form of a plus sign (+). The dimensions of the open and closed arms are 50 *10 cm and there are walls with 40 cm high on both sides and ends of the closed corridor. There is a glass edge mounted on both sides and end of the open corridor at a height of 0.5 cm (12). The four corridors lead to a central area of 10×10 cm. The maze is positioned at a height of 50 cm above the ground by a stand. The mouse is placed within the central range of the maze with an open arm. Suitable light is provided

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by a 60-watt bulb located above the center of the maze (13).

Behavioral test: The animal was kept in the test room for an hour at this stage for familiarization. Each animal was then gently placed in the center of the maze and facing the open arm for 30 min after intraperitoneal nicotine or saline injection. Within 5 minutes, the number of times of entry, duration of presence in the open and closed arms, and motor activity, which represents the total number of times in the open and closed arms were measured.

Then the percentage of open arm entry (%OAE: %Open arm entry), which is equal to the number of times the open arm entry is multiplied by 100, and also the percentage of time left in the open arm (%OAT: %Open arm time)), which is equivalent to the length of time that the open arm was multiplied by the sum of the time multiplied by two arms to 100 were calculated. Significant increase in these two parameters indicates a decrease in anxiety in this test. All sections of the experiment were recorded by a camera located 2.8 meters above ground level just above the center of the maze and connected to a computer (14).

Tissue preparation and immunohistochemistry assay: immunohistochemistry assay was used to evaluate the expression of P-CREB and c-Fos proteins. After anesthesia, intracardiac perfusion was performed using saline phosphate buffer (PBS) followed by 4% paraformaldehyde (PFA: Sigma, USA) in 0.1 mol/L of PBS with pH= 7.4 for the animals. The brains were then removed, fixed in 4% paraformaldehyde overnight, and washed with PBS, tissue sections fixed in paraformaldehyde and paraffin were prepared, tissues were immersed in dehydrated alcohol and immersed in paraffin. Then, microtomes were prepared according to Paxinos and Watson Atlas (10).

The slices were transferred, de-paraffinized, and dehydrated on positive or poly-lysine rechargeable slides. For antigen recovery, citrate buffer was used for P-CREB protein with pH= 9 and for c-fos with pH= 6.8 and the slides were incubated in the microwave for 5 min at high temperature. The internal peroxidase enzyme was incubated by incubating the slices in 3% H2O2 in methanol at room temperature. Blocking protein was used for P-CREB for 15 minutes and for c-fos first proteinase K for 5 minutes and then for 30 minutes were used. Anti-c-Fos (Abcam, ab7963, USA) and anti-P-CREB (Abcam, ab32096, USA) antibodies were diluted one to fifty on the tissues. Slides were washed in PBS and detection solution (envision-TM+Rb) was added to the tissues and the required amount of diaminobenzidine solution (DAB) was poured onto the tissues. Tissues were then mounted with varying degrees of dehydrated alcohol and the cells expressing proteins were counted by light microscopy. The expression level of proteins was rated as 0-3. Protein expression below 5% scored 0, 5% -36% score 1, 37%-68% score 2 and 69%-100% score 3 (15).

Statistical analysis: The data were analyzed using one-way ANOVA test if the data were normal. Following the significance of the results, complementary analysis was performed using Tukey's test to compare the significance of each group with the control group. All evaluations were expressed as Mean±SEM. Immunohistochemical data analysis was performed using non-parametric Kruskal-Wallis test. Following significance, Mann-Whitney U analysis was performed to examine the significance of each group in comparison with the control group. Statistical calculations of the data were performed using SPSS software and graphs were drawn using Sigmaplot software and Graphpad prism software. $P<0.05$ was considered significant.

Results

Behavioral test findings: Significant difference was observed in %OAT ($p<0.001$, $F(2.18)= 13.69$) and %OAE ($p<0.001$, $F(2/18)= 13/41$) between 1mg/kg saline group and different amounts of nicotine 0.3 and 0.7 mg (Figure 1, A and B), but there was not in motor activity ($p>0.05$, $F(2/18)= 51$) (Figure 1, C). Intraperitoneal administration of nicotine 0.7 mg/kg decreased OAT% (5.4±0.42) and OAE% (29.4±0.61) compared to saline (15.2±0.82) and (42.1±0.45) indicating an anxiety-like effect ($p<0.001$ and $p<0.01$, respectively) (Figure 1, A and B).

Immunohistochemical findings of P-CREB: Significant differences was observed in P-CREB protein expression in the BLA area between the test groups ($p<0.05$, $H(3)= 6.99$). P-CREB protein expression in the BLA of control animals (score 3) was higher than the other groups. Nicotine 0.3 mg/kg decreased the expression level of P-CREB protein in the BLA region (score> 1) (Figure 2). The histological

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image of the BLA region is shown (Figure 3). Immunohistochemical images of P-CREB protein expression in the BLA region were seen in control, saline, nicotine 0.3 and 0.7 mg / kg groups (Fig. 4, A, B, C, D, respectively).

Immunohistochemical findings of c-Fos: Significant difference was seen in expression of c-Fos protein in BLA region of experimental groups [p<0.01, H(3)= 13/11]. c-Fos protein expression in the BLA of control animals, nicotine 0.3 and 0.7 mg/kg (score> 1) were significantly lower than the saline group (score 3) (p<0.01) (Figure 5). Immunohistochemical images of c-Fos protein expression in BLA were seen in control, saline, nicotine 0.3 and 0.7 mg/kg groups (Fig. 6, A, B, C, D, respectively).

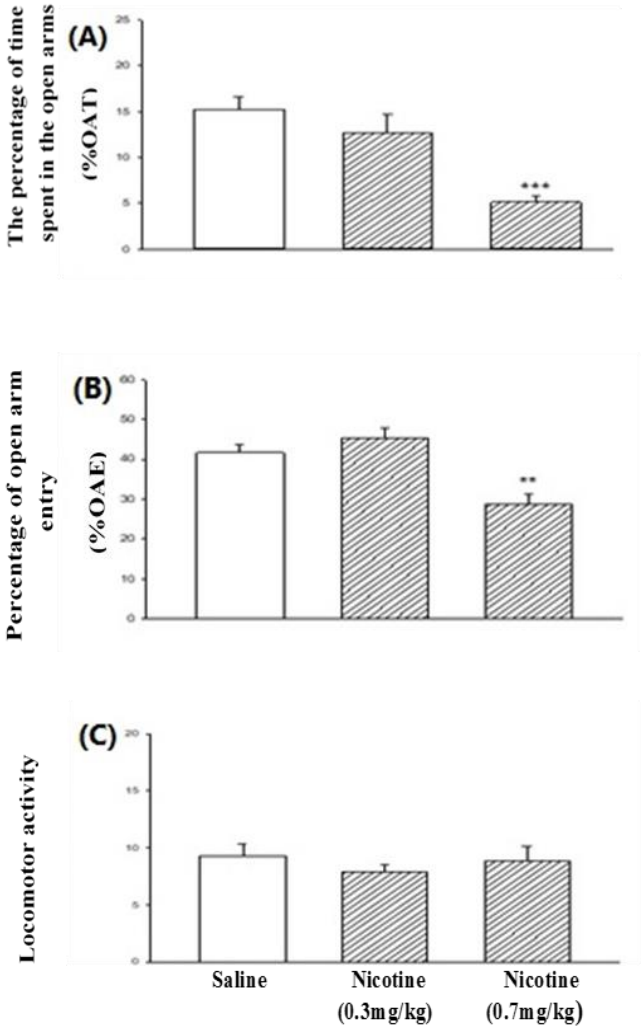


Figure 1. Effect of intraperitoneal administration of nicotine on anxiety-like behavior of animals in EPM ** (p<0.01), * (p<0.001) compared with saline group.**

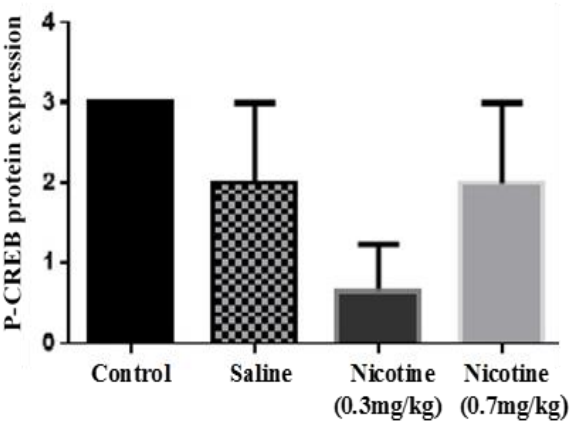


Figure 2. Comparison of P-CREB protein expression in BLA region of experimental groups.

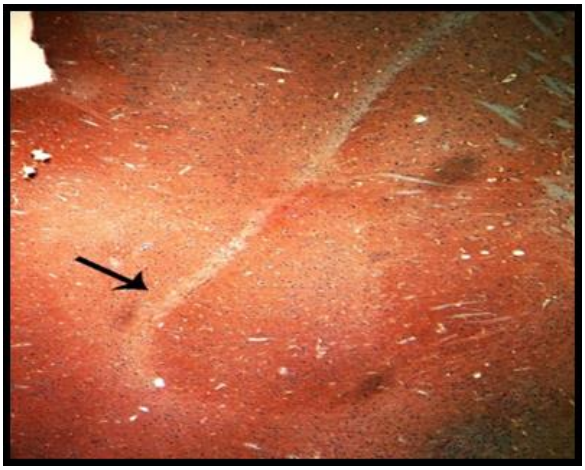


Figure 3. The histological image of the BLA region indicated by an arrow.

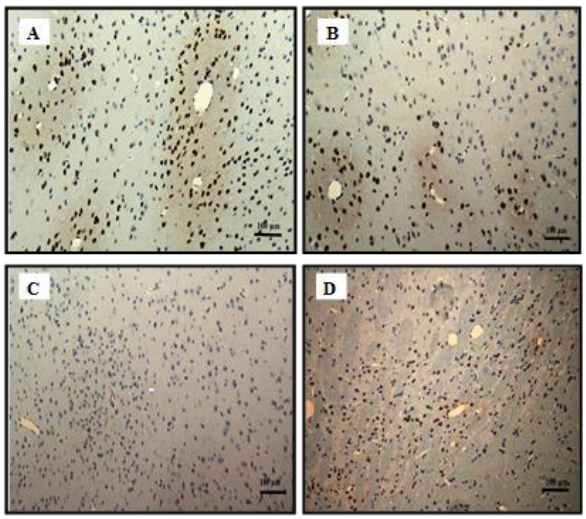


Figure 4. Immunohistochemical images of P-CREB protein expression in BLA. A, B, C and D images of control, saline, nicotine 0.3 mg/kg and nicotine 0.7 mg, respectively).

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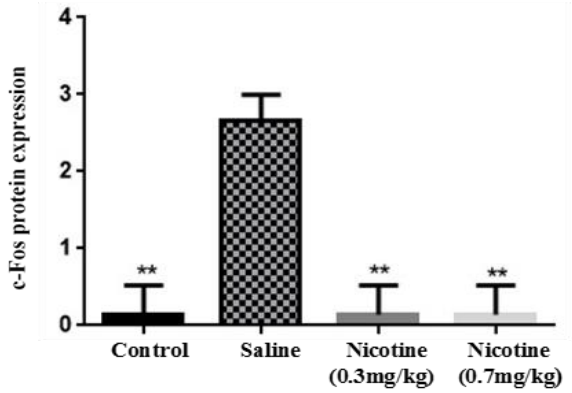


Figure 5. Comparison of c-Fos protein expression in BLA region of experimental groups (p<0.01) ** compared with saline group

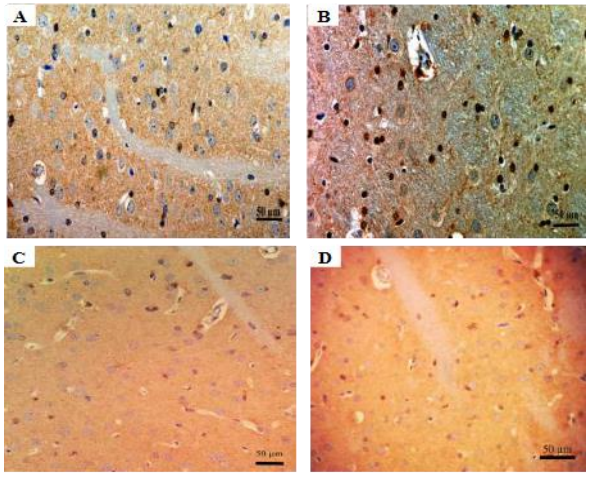


Figure 6. Immunohistochemical images of c-Fos protein expression in BLA. A, B, C and D images are control, saline, nicotine 0.3 mg/kg and nicotine 0.7 mg groups, respectively)

Discussion

In this study, a decrease in %OAT and %OAE following systemic administration of nicotine at dose of 0.7 mg/kg in EPM without any effect on locomotor activity indicated an anxiogenic effect of nicotine. Previous studies have also confirmed the anxiogenic effect of nicotine following systemic administration (16) and central amygdala injection using EPM (17,18). In contrast, some studies have reported nicotine-induced anxiolytic effects in animal models of anxiety (19,20). Nicotine dose, method of administration, animal model used, and breed and species of animal may be influenced by various behavioral responses to the drug. Another important finding from the study was higher levels of P-CREB in control animals than in the other groups. In addition, systemic administration of nicotine at a dose of 0.3 mg/kg reduced the expression of P-CREB protein in the BLA region, whereas a dose of 0.7 mg/kg increased it. Studies have shown that amygdala activity leads to anxiety-related behavioral and physiological responses (21). Rubino et al. reported that p-CREB protein levels in the amygdala did not change after exposure to EPM (22). In another study, CREB activity in the nucleus accumbens decreased as the anxiety-like behaviors increased (23).

Pandey et al. found that decreased CREB function in the amygdala correlated with anxiety-like behavior in rats who consumed alcohol (24). Studies have shown that acute administration of nicotine had no effect on the overall levels of CREB and p-CREB in the nucleus accumbens, but chronic administration of nicotine significantly decreased the levels of these proteins (25). Another study observed that when the α-2-adrenoceptor agonist was administered prior to training, a decrease in CREB phosphorylation and c-Fos expression occurred in the central, basolateral, and lateral nuclei of the amygdala (7). The brain region under study, the type of behavioral test, and the drug being administered may be the cause of the different responses in the studies. The results also showed that the animals receiving saline expressed a higher level of c-Fos than the other groups. In support of our findings, there are reports that c-Fos expression in the amygdala increases after exposure to EPM (22,26). In addition, the findings of the present study showed that systemic administration of nicotine decreases c-Fos protein expression in the BLA region. Kiba et al reported that acute injections of nicotine in rats lead to increased c-Fos expression in the striatum, which is mediated through dopamine receptors (27).

Another study showed that after systemic administration of nicotine, brain areas such as the thalamus and ventral tegmental area did not express c-Fos (28). Chen et al. showed that nicotine regulates anxiety-like behavior and c-Fos expression in mice. These subjects observed that anxiety led to a significant increase in c-Fos expression in the central and basolateral nucleus of the amygdala (29). These observations suggest that a subset of nervous system neurons are involved in nicotine behavioral responses to changes in c-Fos expression. Drug dose, method of administration, schedule of administration, and brain

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area studied play important roles in c-Fos protein expression in investigations. Since in the present study, alterations in the expression of c-Fos and P-CREB proteins were observed in different treatment groups underwent EPM, it can be concluded that the BLA region can partially play a role in the anxiogenic effects of nicotine.

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