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Stress induces behavioral sensitization, increases nicotine-seeking behavior and leads to a decrease of CREB in the nucleus accumbens

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ABSTRACT

Experimental evidence shows that exposure to stress engenders behavioral sensitization and increases drug-seeking and leads to intense drug taking. However the molecular mechanisms involved in these processes is not well known yet. The present experiments examined the effects of exposure to variable stress on nicotine-induced locomotor activation, cAMP-response element-binding protein (CREB) and extracellular signal-regulated kinase (ERK) activity and nicotine intravenous self-administration in rats. Male Wistar rats were exposed to variable stress that consisted of the exposure to different stressors twice a day in random order for 10 days. During this period the control group was left undisturbed except for cage cleaning. Ten days after the last stress episode, rats were challenged with either saline or nicotine (0.4 mg/kg s.c.) and the locomotor activity was recorded for 20 min. Immediately after behavioral recordings rats were sacrificed and their brains were removed to posterior western blotting analysis of CREB, phosphoCREB, ERK and phosphoERK in the nucleus accumbens. An independent set of control and stressed animals were subjected to an intravenous nicotine self-administration protocol. The break point during a progressive ratio schedule and nicotine intake patterns during a 24-hour binge was analyzed. Repeated variable stress caused a sensitized motor response to a single challenge of nicotine and decreased CREB in the nucleus accumbens. Furthermore, in the self-administration experiments previous stress exposure caused an increase in the break point and nicotine intake.

1. Introduction

Several data point to a positive correlation between exposure to stress and increased vulnerability to drug addiction (Dembo et al., 1988; Gordon, 2002; Koob, 2008; Perkins, 1999; Sinha, 2001, 2008). In this sense, exposure to stress appears to increase the number of cigarettes smoked and is strongly associated with craving and relapse to tobacco smoking (Cohen and Lichtenstein, 1990; Kassel et al., 2003; Niaura and Abrams, 2002).

Pre-clinical studies have demonstrated that stress facilitated the acquisition of drug self-administration and promoted reinstatement of drug-seeking (Cruz et al., 2010; Haney et al., 1995; Kabbaj et al., 2001; Leão et al., 2009; Shalev et al., 2002). Several studies have demonstrated that rodents exposed to repeated stress procedures such as restraint, footshock or social defeat, displayed increased cocaine-taking and -seeking behaviors (Covington and Miczek, 2005; Piazza et al., 1990; Quadros and Miczek, 2009; Shaham and Stewart, 1994). Moreover, it has been shown that exposure to four social defeat sessions promoted escalated cocaine self-administration and increased the break-point on progressive ratio sessions (Covington and Miczek, 2001; Cruz et al., 2011). Although the interplay between exposure to stress and cocaine self-administration has been well characterized, little is known about how repeated stress changes nicotine self-administration.

Repeated administration of drugs of abuse induces behavioral sensitization, as evidenced by an enhanced locomotor response to a subsequent injection of the drug (Clarke and Kumar, 1983; Domino, 2001; Kita et al., 1999; Shim et al., 2001). Similarly, repeated exposure to stress also induces augmented sensitivity to drug-induced hyperactivity that has been termed "cross-sensitization" between stress and drug (Miczek et al., 2008). Behavioral sensitization has been suggested as an animal model of neuroplasticity associated mainly with the development of drug addiction (Robinson and Becker, 1986; Robinson and Berridge, 1993; Vanderschuren and Kalivas, 2000) and drug-induced psychosis (Robinson and Becker, 1986). Although nicotine causes behavioral sensitization in rats (Cruz et al., 2005; Faraday et al., 2005), stress-induced cross-sensitization to nicotine remains controversial and seems to depend on intensity, duration or frequency of stressful events (Cruz et al., 2008; Kita et al., 1999). For example, Kita et al. (1999) demonstrated that rats subjected to repeated exposure to social stress displayed enhanced nicotine-induced behavioral sensitization. However, a study of our laboratory showed that previous exposure to restraint stress for 7 days did not affect nicotine-induced locomotor-activating effects in adult and adolescent rats (Cruz et al., 2008).

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Studies in animals have demonstrated that stress-induced cross-sensitization to psychostimulants might facilitate acquisition of cocaine self-administration and increase the break-point and cocaine intake during a 24-hour "binge" session (Covington and Miczek, 2001, 2005; Covington et al., 2008). Although prior exposure to stress, particularly when inducing sensitization, influences subsequent drug taking behavior (Covington and Miczek, 2001; Piazza et al., 1990), little is known about the interplay between sensitization to nicotine and its self-administration.

Stress-induced locomotor sensitization and escalated psychostimulant self-administration has been related to neuroplasticity in mesolimbic system (Nestler, 2005; Robinson and Berridge, 1993). Evidence points that changes in the expression and activity of cAMP-response element-binding protein (CREB) and extracellular signal-regulated kinase (ERK) are involved in the development of behavioral sensitization and increased drug-taking behavior (Lu et al., 2006; Mattson et al., 2005; Pierce and Kalivas, 1997).

Recently, it has shown that repeated administration of cocaine enhanced the amount of phosphorylated ERK and CREB in the nucleus accumbens (NAc) (Berhow et al., 1996; Mattson et al., 2005). In contrast, chronic nicotine treatment decreased phosphorylated CREB in the NAc and this effect persisted at least for 18 h (Pandey et al., 2001; Pluzarev and Pandey, 2004). Exposure to stress is also related to changes in CREB and ERK activity. In this sense, Muschamp et al. (2011) demonstrated that the exposure to footshock stress enhanced CREB activity within the nucleus accumbens shell. In addition, Bruchas et al. (2008) reported that exposure to repeated forced swim stress caused a significant increase in phosphorylation of ERK in both the caudate and NAc regions of the mouse striatum. However changes in ERK and CREB pathways and its relation to the interplay between the exposure to stress and changes in nicotine effects have been poorly investigated.

Thus, the present study investigated the consequences of repeated exposure to variable stress on nicotine-induced locomotor activity and self-administration. The progressive-ratio schedule was used to assess possible elevations in the break-point parameter to receive nicotine. In addition, a 24-hour "binge" was employed to measure nicotine intake under conditions of prolonged access. In addition, we investigated whether changes in ERK and CREB activity in the NAc could be involved in stress-induced changes in nicotine behavioral effects.

2. Methods

2.1. Subjects

Male Wistar rats, 225–250 g at arrival, obtained from the animal breeding facility of the Univ. Estadual Paulista—UNESP were individually housed in plastic cages 19 cm (width) × 30 cm (length) × 14 cm (height).

Rats were continuously maintained on a reversed light cycle (12-hour:12-hour, lights off at 08:00 am) with controlled temperature (21 °C) and humidity (35–40%). During the entire nicotine self-administration protocol rats received 18 g rat chow per day provided in their home cage after each daily experimental session during the entire self-administration protocol. This feeding schedule resulted in a gradual weight gain of approximately 15 g/week. This observation is in line with other studies which have shown that the acquisition and maintenance of nicotine self-administration is extremely sensitive to conditions of feeding, and weight gain (Lang et al., 1977; Corrigall and Coen, 1989; Donny et al., 1995). Unlimited access to water was available throughout all experiments. All experiments were performed during the dark phase.

The experimental protocol was approved by the Ethics Committee for Use of Human or Animal Subjects of the School of Pharmaceutical Science, UNESP (CEP-19/2008) and the experiments were conducted according to ethics principles of the Colégio Brasileiro de Experimentação Animal, based on the NIH Guidelines for the Care and Use of Laboratory Animals.

2.2. Chronic stress

The chronic variable stress protocol, modified from Ortiz et al. (1996), consisted of exposure to different stressors twice a day for 10 days (Table 1). All stress sessions were performed in an adjacent room to the animal's facility. The control group was left undisturbed except for cleaning the cages. Ten days after the last stress session the animals were subjected either to the locomotor test or to the self-administration protocol.

2.3. Locomotor response to nicotine

2.3.1. Apparatus

Locomotor activity measures were conducted in commercially available (Columbus Instruments, Columbus, OH, USA) activity monitoring chambers, consisting of Plexiglas cages. The chambers, measuring 44 cm (width) × 44 cm (length) × 20 cm (height) cm included 10 pairs of photocells beams, which were used to measure the horizontal locomotor activity. The consecutive interruption of two beams was recorded as one locomotor count.

2.3.2. Locomotor measurement

Ten days after the last exposure to stress, animals were injected with a challenge dose of saline (1.0 ml/kg s.c.) (control n = 10; stress n = 11) or nicotine (0.4 mg/kg s.c.) (control n = 10; stress n = 10). Immediately following the injections, animals were put in an activity chamber and their locomotor activity was recorded during a 20-minute testing session as described above. Animals were allowed a 20-minute habituation period to the photocell apparatus immediately prior to injections. Animals from different groups were tested randomly during the dark phase between 10:00 am and 14:00 pm.

2.4. Molecular analysis

2.4.1. Collection of brains

Immediately after locomotor test brains were extracted and frozen in isopentane (−50 °C) within 30–45 s of decapitation and stored at −80 °C until dissection.

Table 1
Chronic variable stress protocol, consisting of exposure to different stressors twice a day for 10 days.

Day	Stress procedure	
1st day	Restraint for 1 h (dark phase)	Wet bedding for 12 h (light phase)
2nd day	Cold exposure (4 °C) for 1 h (dark phase)	Turn off the lights for 12 h (light phase)
3rd day	Turn on the lights for 3 h (dark phase)	Forced swim for 4 min (dark phase)
4th day	Food and water deprivation for 12 h (dark phase)	Stroboscopic light for 12 h (light phase)
5th day	Forced swim for 3 min (dark phase)	Wet bedding for 12 h (dark phase)
6th day	Restraint for 1 h (dark phase)	Food and water deprivation for 12 h (dark phase)
7th day	Turn on the lights for 2 h (dark phase)	Wet bedding for 12 h (light phase)
8th day	Cold exposure (4 °C) for 15 min (dark phase)	Turn off the lights for 12 h (light phase)
9th day	Restraint for 1 h (dark phase)	Forced swim for 4 min (dark phase)
10th day	Stroboscopic light for 12 h (dark phase)	Food and water deprivation for 12 h (dark phase)

2.4.2. Western blotting analysis of CREB, phosphoCREB, ERK and phosphoERK

One-millimeter coronal slices of brain (rostral face approximately 2.0 mm from Bregma based on the atlas of Paxinos and Watson (2005)) were cut in a cryostat at −20 °C. Tissue punches (blunt 14-gauge needle) were obtained from nucleus accumbens (see Fig. 1) and then sonicated in 1% sodium dodecyl sulfate (SDS). Tissues were kept frozen until sonication in SDS. Protein concentrations of the samples were determined using the method of Lowry (Bio-Rad Laboratories, Hercules, CA, USA). Sample protein concentrations were equalized by diluting with 1% SDS. Samples of 30 μg of protein were then subjected to SDS-polyacrylamide gel electrophoresis for 3 h at 200 V. Proteins were transferred electrophoretically to polyvinylidene fluoride (PVDF) membrane for immunoblotting (Hybond LFP transfer membrane—GE Healthcare, UK) at 0.3 Å for 3.5 h. Then PVDF membranes were blocked with 5% nonfat dry milk and 0.1% Tween 20 in Tris buffer (TTBS, pH 7.5) for 1 h at room temperature and then incubated overnight at 4 °C in fresh blocking buffer (2% nonfat dry milk and 0.1% Tween 20 in Tris buffer (TTBS, pH 7.5)) containing two primary antibodies for each signaling protein: one antibody specifically labeled the phosphorylated form of the protein while the second antibody labeled non-phosphorylated (total CREB) of the same protein. Each pair of antibodies was chosen so that one was a rabbit antibody while the other was a mouse antibody. CREB phosphorylation levels were assessed using antibodies against total CREB (1:500; Cat# 9197; Cell Signaling Technology, Danvers, MA, USA) and phosphoCREB (1:1000; Cat# 05–807; Millipore Corporation, Billerica, MA, USA). ERK activity levels were assessed using antibodies against total ERK (1:2000; Cat# 9102; Cell Signaling Technology, Danvers, MA, USA) and phospho ERK (1:1000; Cat# 7383; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with primary antibodies, blots were washed and incubated for 1 h with anti-rabbit secondary antibodies labeled with fluorophor (ECL Plex, GE Heathcare®, Pittsburgh, PA, USA) (Cy5-anti-rabbit/1:3000 and Cy3-anti-mouse/1:5000). Fluorescence was assessed using a fluorescence scanner (Typhoon—GE Healthcare®, Pittsburgh, PA, USA), and bands were quantified using suitable software (Image Quant™—GE Heathcare®, Pittsburgh, PA, USA).

Equal protein loading was confirmed by stripping the blots and re-probing them with a monoclonal actin antibody (1:500; Cat# A5316 Sigma-Aldrich, St. Louis, MO, USA), followed by incubation with respective secondary antibody (Cy5-anti-rabbit/1:3000) and visualization as described above.

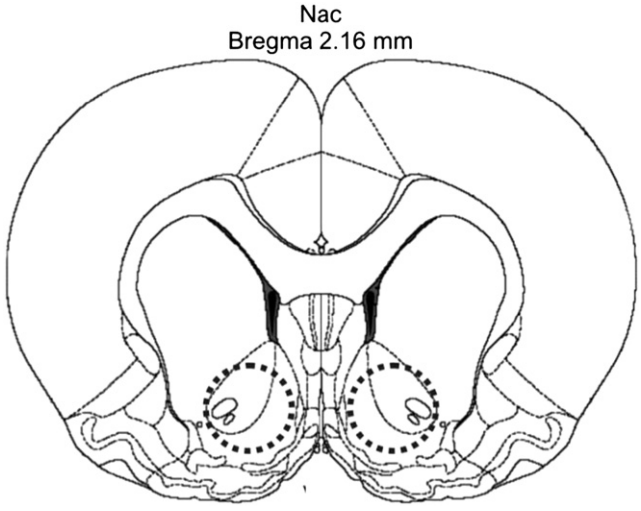

Fig. 1. Schematic sections of the rat brain, adapted from the stereotaxic atlas of Paxinos and Watson (2005), showing the location of punches in the nucleus accumbens (NAcc).

2.5. Intravenous drug self-administration

The general procedure to establish nicotine self-administration was adapted from George et al. (2007).

2.5.1. Apparatus

For nicotine self-administration the animals were put individually in Plexiglas experimental chambers (30×30.5×24.5 cm), enclosed in light- and sound attenuating boxes. The floor of the chambers consisted of a Plexiglas tray covered with sawdust. A hole in the ceiling allowed the passage and free movement of the tethered catheter (Strategic Applications Inc., Libertyville, IL, USA) that was connected to a counterbalanced swivel and an infusion pump (Insight Equipments®, Ribeirão Preto, SP, Brazil). The front wall of the chamber contained one interchangeable panel (Insight Equipments®, Ribeirão Preto, SP, Brazil). The panel was equipped with two levers, located 5 cm from the floor, two cue lights (red and green) above each lever and a session light in the middle of the panel (12 cm from the floor).

2.5.2. Drug

Nicotine 99% was obtained from Sigma-Aldrich (St Louis, MO, USA). The dose of nicotine was chosen based on previous experiments conducted in our laboratory.

2.5.3. Training

Ten days after the last exposure to stress, animals were subjected to the training sections. Training consisted of three 60-minute sessions, in which each response on the active lever (alternated between left and right sides) was reinforced with the delivery of 0.2 ml sucrose (6%) (fixed ratio schedule of reinforcement; FR 1), followed by a 10-second time-out. Each rat was allowed continuous access to sucrose solution during the entire 60 min. Responding on the inactive lever had no scheduled consequence. Rats were deprived of water for 8 h before the beginning of the first sucrose training session.

2.5.4. Surgery

Twenty-four hours after the last training session, stressed and control rats were implanted with permanently indwelling catheters (Silastic™ silicon tubing, inner diameter = 0.63 mm, outer diameter = 1.17 mm) into the right jugular vein under a combination of ketamine (100.0 mg/kg) and xylazine (6.0 mg/kg) anesthesia. The catheter was passed subcutaneously to the rat's back where it exited through a small incision and was affixed to a plastic pedestal (Strategic Applications Inc., Libertyville, IL, USA) mounted inside a harness system (Strategic Applications Inc., Libertyville, IL, USA). Rats were allowed to recover from surgery for 5 days in their home cage with free access to food and water. To prevent inflammation and infection rats received cetoprophen 1% (5.0 mg/kg; i.m.), and cefazolin (10.0 mg/kg; i.v.) for three consecutive days following surgery.

The catheter was flushed daily with heparinized saline (20 IU/ml) and 0.2 ml of saline in order to maintain the catheter's patency.

2.5.5. Nicotine self-administration

After recovering from surgery all rats were initially given unlimited access to sucrose (6%) self-administration, in this procedure each lever press was reinforced with the delivery of 0.2 ml sucrose (6%) (fixed ratio schedule of reinforcement; FR 1), followed by a 10-second time-out. During self-administration sessions, a green cue light above the active lever signaled sucrose availability and a red cue light, also above the active lever, signaled sucrose delivery. During the time-out period, the green and red cue lights were extinguished and lever press were recorded but had no consequences. Each daily session was terminated after 60-minute of access. After three sessions of sucrose reinforcement nicotine self-administration was assessed during 3-hour sessions. Initially, nicotine (0.03 mg/kg per infusion) was infused (0.1 ml/s)

associated to sucrose 6% (0.2 ml v.o.) delivered on schedule of reinforcement (FR1) for the active lever. Responses on the inactive lever were recorded but had no programmed consequence. Each daily session was terminated after 10 drug infusions or 3 h of access, whichever occurred first. Simultaneous administration of sucrose and nicotine sessions was ended after completing 10 drug infusions within 3 h over two consecutive days. Following this period only nicotine (0.03 mg/kg per infusion) was infused (0.1 ml/s) on schedule of reinforcement (FR1) for the active lever. After completing 10 infusions of nicotine within 3 h over two consecutive days, the FR schedule was progressively increased to fixed ratio 3 (FR 3). Rats were maintained for at least four additional days on a limited access on FR 3 schedule before being examined during a progressive ratio schedule of reinforcement.

2.5.6. Progressive ratio schedule of drug reinforcement

After the acquisition and maintenance phase, self-administration according to a progressive ratio (PR) schedule of drug reinforcement was verified. The progression of response requirements followed the algorithm used in Hodos (1961): 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26… The final infusion delivered was defined as the break point. The PR session was terminated once the rat failed to obtain an infusion during 60 min. The average number of total responses and the last ratio completed across three PR trials for each individual rat was calculated.

2.5.7. 24-Hour unlimited access drug "binge"

After the final PR session, each rat was allowed one additional day of limited drug access (0.03 mg/kg per infusion of nicotine, FR3 schedule, total of 10 infusions). The very next day, a 24-hour binge protocol was implemented starting at 10:00 am. Each rat was allowed continuous access to drug infusions (0.03 mg/kg per infusion—0.1 ml/s) on FR1 schedule during the entire 24-hour binge. The amounts of drug self-administered as well as the pattern of responding were recorded. Lights were off during the whole binge session (Covington and Miczek, 2001). A bottle of water and 18 g rat chow were put in self-administration boxes in the beginning of the binge session.

2.6. Statistics

Data from the locomotor activity and molecular analysis were analyzed by two-way ANOVA considering group (stress vs control) and treatment (saline vs nicotine) factors. Newman–Keuls' test was employed for individual post-hoc comparisons.

Nicotine self-administration data were analyzed using Student's *t*-tests comparing stress and control groups. Significant differences are reported for $p<0.05$.

3. Results

3.1. Locomotor activity

Fig. 2 depicts the locomotor activity following a challenge injection of nicotine or saline, 10 days after the last exposure to variable stress.

Two-way ANOVA revealed significant differences in locomotor activity considering group [$F(1,33)=5.4; p<0.05$] and treatment factors [$F(1,33)=5.96; p<0,05$]. No significant interaction between factors was detected [$F(3,33)=1.13; p>0.05$]. Unprotected comparisons by Newman–Keuls test was performed, which revealed that stress-nicotine group showed higher locomotor activity when compared to the other groups, $p<0.05$, revealing that exposure to variable stress promoted behavioral cross-sensitization to nicotine.

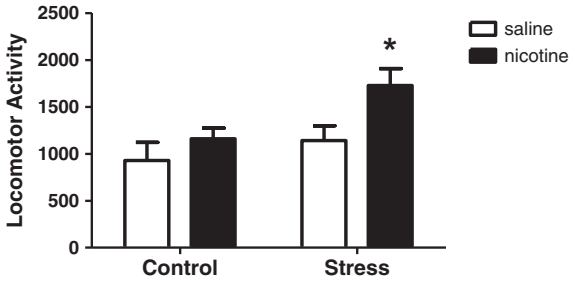

Fig. 2. Locomotor activity following saline or nicotine (0.4 mg/kg i.p.) challenge injections in rats exposed to chronic variable stress. Data represent mean ± SEM (N = 10–11 animals per group) of cumulative locomotion counts recorded for 20 min immediately after injections. *$p<0.05$ stress-nicotine group when compared to other groups.

3.2. Sucrose self-administration

Fig. 3 shows the daily sucrose consumption (3 days) in the stress and control group.

There were no significant differences for group or days factors [$F(3,33)=0.44; p>0.05$ and $F(3,33)=0.67; p>0.05$, respectively]. No significant interaction between factors was detected [$F(3,33)=0.03; p>0.05$].

3.3. Nicotine self-administration

3.3.1. Progressive ratio schedule

Fig. 4 shows the number of responses (A) and the last ratio achieved (B) in a progressive ratio schedule of control and stress group.

Student's *t*-test revealed a significant increase in the number of responses in the stress when compared to the control group ($t_{(15)}=4.346; p<0.001$). In addition, the final ratio achieved was higher in the stress group as compared to the control one ($t_{(15)}=3.4; p<0.05$) (Fig. 4).

3.3.2. Twenty-four hours unlimited access drug "binge"

Fig. 5 shows individual (A) and the mean (B) of total nicotine intake during 24-hour "binge" session of control and stressed animals.

Animals exposed to variable stress showed higher nicotine intake during a 24-hour "binge" session when compared to the control group ($t_{(13)}=1.875; p<0.05$; Student's *t*-test).

3.4. Western blotting analysis of CREB and ERK activity

3.4.1. CREB activity

Fig. 6 shows the western blotting analysis of total CREB and phospho-CREB in the nucleus accumbens of samples collected after locomotor activity tests.

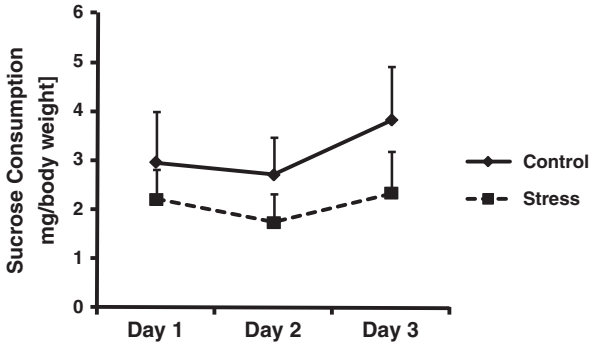

Fig. 3. Sucrose consumption of control or variable stress groups (mean ± SEM of 9 animals per group).

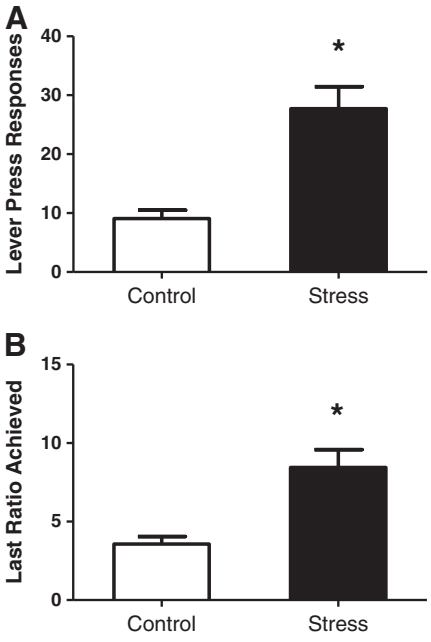

Fig. 4. Number of responses (A), and last ratio achieved (B) of nicotine (0.03 mg/kg per infusion; i.v.) in a progressive ratio schedule. Data represent mean ± SEM of N = 8 animals per group. *p<0.05 compared to the control group (Student's *t*-test).

For total CREB, ANOVA indicated a main effect for the stress [$F_{(1,25)} = 7.73; p < 0.01$], but not for the treatment factor [$F_{(1,25)} = 0.23; p > 0.05$]. No interaction between factors was detected [$F_{(1,25)} = 0.4; p > 0.05$].

Concerning phosphoCREB, ANOVA showed significant differences for stress [$F_{(1,26)} = 8.60; p < 0.01$] and treatment factors [$F_{(1,26)} = 6.70; p < 0.01$]. However, significant interaction between factors was not observed [$F_{(1,25)} = 3.37; p > 0.05$]. Unprotected Newman–Keuls post hoc test indicated that nicotine administration decreased phospho-CREB levels in the control group ($p < 0.05$). In general, exposure to stress per se decreased total CREB and consequently CREB phosphorylation levels.

3.4.2. ERK activity

Fig. 7 shows the western blotting analysis of total ERK and phosphoERK in the nucleus accumbens of samples collected after locomotor activity test.

Two-way ANOVA of total ERK did not reveal significant differences for stress [$F_{(1,36)} = 0.47; p > 0.05$] or treatment factors [$F_{(1,36)} = 0.11; p > 0.05$]. No interaction was observed between the factors [$F_{(1,36)} = 0.05; p > 0.05$].

Concerning phosphoERK, ANOVA did not show significant differences for stress [$F_{(1,36)} = 2.62; p > 0.05$] or treatment factors [$F_{(1,36)} = 0.19; p > 0.05$]. No interaction was observed between the factors [$F_{(1,36)} = 0.002; p > 0.05$].

4. Discussion

The findings of the present study suggest that exposure to chronic variable stress promotes long-lasting behavioral consequences as well as neural plasticity that can be related to enhanced nicotine self-administration. Specifically, exposure to variable stress promoted locomotor sensitization to nicotine and increased patterns of nicotine self-administration when assessed by progressive ratio and escalated intake of nicotine during the unlimited access binge. Additionally, exposure to variable stress reduced total CREB and phosphoCREB within the nucleus accumbens. To our knowledge this is the first study on the interplay between neural plasticity induced by repeated exposure to stress and nicotine self-administration.

Our findings on cross-sensitization between variable stress and nicotine are in accordance with a previous study showing that exposure to psychological stress daily, for 10 days, caused sensitization to nicotine-induced ambulatory stimulation (Kita et al., 1999). In contrast, other studies have demonstrated that exposure to repeated restraint stress did not promote cross-sensitization to nicotine (Cruz et al., 2008; Faraday et al., 2003; McCormick and Ibrahim, 2007). These data suggest that stress-induced sensitization to the locomotor effects of nicotine might depend on kind and intensity of the stress regimen. Our results also corroborate studies for other substances demonstrating that chronic exposure to stress enhanced the locomotor response to a subsequent drug administration. For instance, cross-sensitization to psychostimulants has been observed after repeated footshock (Kalivas and Duffy, 1989; Sorg and Kalivas, 1991), restraint (Araujo et al., 2003; Hahn et al., 1986), food restriction (Cabib and Puglisi-Allegra, 1994), and social defeat stress (Covington and Miczek, 2001; Yap and Miczek, 2007).

Behavioral sensitization has been proposed as an animal model that may reflect molecular changes in mesocorticolimbic system related to drug addiction (Berridge and Robinson, 1998; Robinson and Berridge, 1993). The mesocorticolimbic pathway is involved with many complex drug responses such as psychomotor and motivated behavioral responses (Everitt and Robbins, 2005; Robinson and Berridge, 2003). Thus, changes in this system might cause locomotor sensitization and alterations in motivated responses as well (Robinson and Berridge, 2003; Berridge, 2007). To address this issue we evaluated the effects of stress on nicotine-seeking behavior through intravenous self-administration, which is considered the most reliable and predictive experimental model for evaluation drug-reinforcing effects in animals (Panlilio and Goldberg, 2007). Although this method is considered a

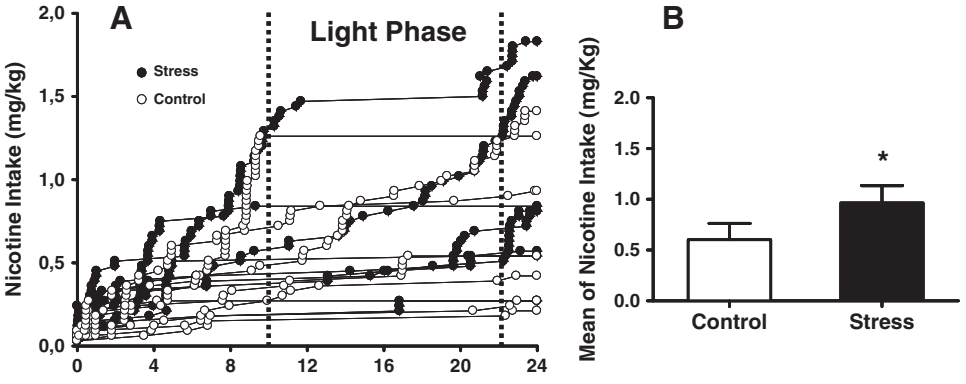

Fig. 5. Individual intake of nicotine (A) (0.03 mg/kg per infusion; i.v.) of control or stress groups; total nicotine intake (mg/kg) during a 24-hour binge session (B). Data represent mean ± SEM of 8–9 animals per group. *p<0.05 compared to the control group (Student's *t*-test).

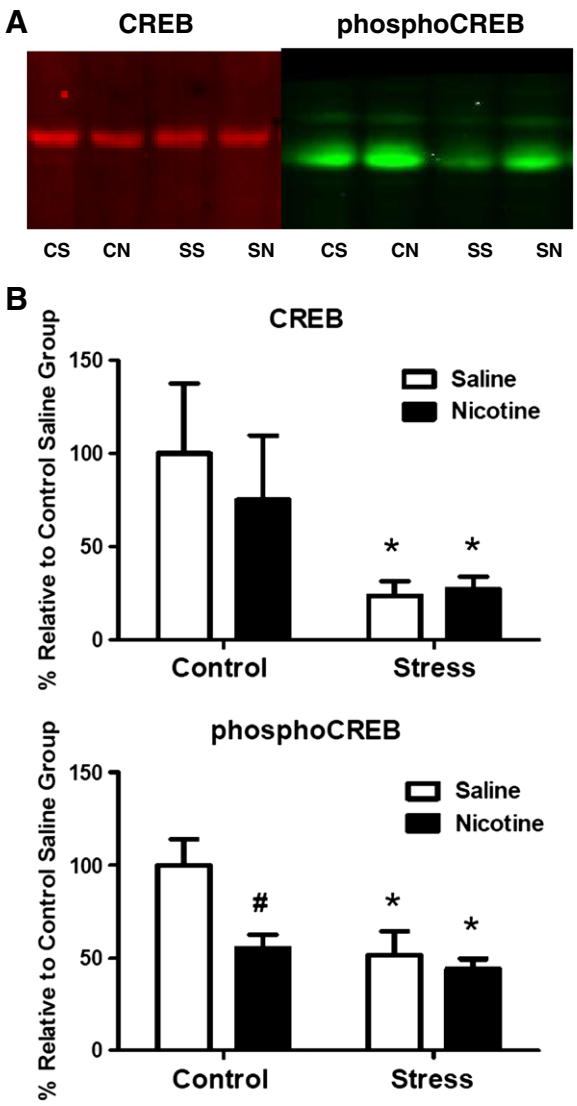

Fig. 6. (A) Representative membrane with fluorescent bands from Western blots of CREB and phosphoCREB (CS: control-saline; CN: control-nicotine; SS: stress-saline; SN: stress-nicotine). (B) Western blotting analysis of total CREB and phosphoCREB in the nucleus accumbens in samples collected after locomotor activity tests. Data represent ±SEM of 9–10 animals per group. *$p<0.05$ when compared to the control saline group; #$p<0.01$ when compared to the control saline group.

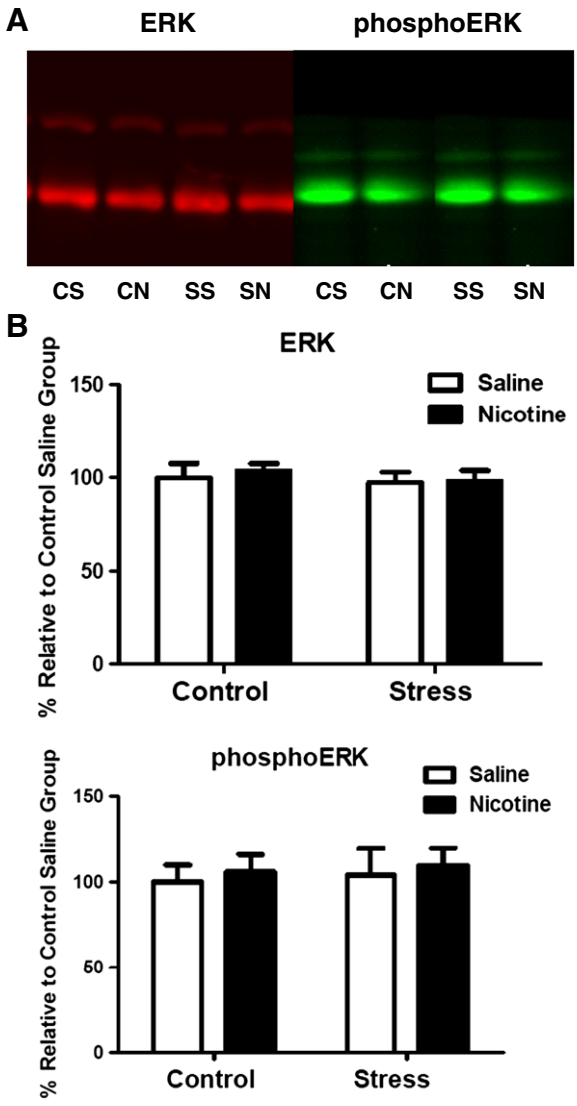

Fig. 7. (A) Representative membrane with fluorescent bands from Western blots of ERK and phosphoERK (CS: control-saline; CN: control-nicotine; SS: stress-saline; SN: stress-nicotine). (B) Western blotting analysis of total ERK and phosphoERK in the nucleus accumbens in samples collected after locomotor activity tests. Data represent ±SEM of 9–10 animals per group.

model with a high face and predictive validity (Clemens et al., 2010; Thomsen and Caine, 2007), the nicotine self-administration protocol is difficult to perform because the reinforcing effect of this drug is relatively lower compared to other drugs of abuse (Caggiula et al., 2002; Donny et al., 1995, 2000). To obtain a robust pattern of nicotine self-administration the association between environmental cues and lever pressing is strictly necessary (Caggiula et al., 2002). We used a protocol in which each nicotine reinforcement was paired to an environmental cue (light). Using this procedure our animals showed a pattern of nicotine self-administration similar to that observed by Clemens et al. (2010) and Wouda et al. (2011).

In the self-administration protocol the break-point under PR schedule presumably reflects the motivation of the animal to self-administer a drug (Depoortere et al., 1993; Hodos, 1961; Richardson and Roberts, 1996). In our study, the PR schedule revealed a significant increase in the break-point in stress-sensitized rats relative to controls, suggesting that exposure to stress increases the motivation to nicotine self-administration. These data are consistent with other findings showing that exposure to four episodes of defeat stress produces behavioral sensitization and increases the break-point to obtain cocaine during a PR schedule (Covington and Miczek, 2001). Similarly, it was demonstrated that rats exposed to footshock stress displayed augmented PR break points for heroin relative to their controls (Shaham and Stewart, 1994).

Our protocol of prolonged access (binge) to nicotine self-administration produced a robust cumulative intake (24 h) (0.6 mg/kg) that was similar to data reported in other studies (0.18–1.5 mg/kg/day) (Caggiula et al., 2001, 2002; LeSage et al., 2003). Using this protocol we demonstrated that exposure to variable stress increased nicotine-seeking behavior assessed during a 24-hour binge session. These results corroborate with others showing that previous exposure to stress increase cocaine and amphetamine intake (Covington and Miczek, 2005; Goeders and Guerin, 1994; Haney et al., 1995; Miczek and Mutschler, 1996; Piazza et al., 1990; Shaham and Stewart, 1994). Our findings are also in accordance with clinical data showing that stress exposure increases the number of cigarettes smoked (Cohen and Lichtenstein, 1990; Kassel et al., 2003; Niaura et al., 2002).

It is important to note that the relationship between stress-induced sensitization to nicotine and increased nicotine self-administration has been poorly investigated. The lack of literature limits further discussions. Most of studies cited above concerns to psychostimulants. However, in

spite of the similar behavioral responses of nicotine and psychostimulants to stress, we should consider that nicotine has a different mechanism of action on the dopaminergic system (Barik and Wonnacott, 2009).

Concerning sucrose consumption no significant differences were observed in the sucrose intake between stress and control groups. Thus, these results suggest that sucrose did not influence the acquisition of nicotine self-administration. Moreover, our results show that stress did not alter the consumption of the natural reinforce, suggesting that stress promotes changes in mesolimbic system that could be specific to drugs.

Long-lasting stress-induced sensitization to drugs and increased drug self-administration, as demonstrated in our study, has been associated with enduring changes in central dopaminergic system (del Rosario et al., 2002; Miczek et al., 2008; Paulson et al., 1991; Rougé-Pont et al., 1995; Yap et al., 2006). Indeed, it has been demonstrated that exposure to chronic stress induces several neuroadaptations in the NAc (Cadoni et al., 2003; Der-Avakian et al., 2006; Miczek et al., 2008; Prasad et al., 1995; Shirayama and Chaki, 2006). For instance, Miczek et al. (2011) showed that rats exposed to intermittent sessions of social defeat stress displayed a sensitized dopamine response to stress in the nucleus accumbens.

Stress can also alter the activity of CREB (via phosphorylation) in the NAc and this may affect motivated behaviors (Carlezon et al., 1998; Pliakas et al., 2001). Both increase and decrease in CREB activity in nucleus accumbens has been observed depending on the stress protocol (Barrot et al., 2005; Muschamp et al., 2011). Pliakas et al. (2001) showed that forced swim stress increase CREB in the NAc shell. In contrast, Wallace et al. (2009) showed that the exposure to social isolation stress leads to decreases in CREB activity within the NAc shell. Our results demonstrated that exposure to variable stress decreased total CREB and consequently CREB activity (evidenced by a reduction of phosphoCREB) in the nucleus accumbens. These results corroborate others showing that exposure to chronic unpredictable stress leads to decreases in phosphoCREB in the striatum (Laifenfeld et al., 2005; Trentani et al., 2002). However, it is important to point out that total CREB levels were also diminished by stress, suggesting changes in the CREB gene expression.

Decreased CREB activity in the nucleus accumbens has been associated with enhanced drug reward (Briand and Blendy, 2010; Carlezon et al., 1998). For example, it was reported that elevated CREB in the NAc impairs cocaine- and morphine-induced conditioned place preference (Barrot et al., 2002; Carlezon et al., 1998). Moreover, increased CREB activity in the striatum has also been related to a decrease in motivation of rats to self-administer cocaine (Hollander et al., 2010). Conversely, Barrot et al. (2002) showed that elevated CREB in the NAc reduced the sensitivity to rewarding brain stimulation. Taken together, our results suggest the increase in break-point and escalation of nicotine self-administration in stressed animals might be related to the decrease in CREB activity in the NAc observed after exposure to variable stress.

We also observed that acute nicotine administration reduces CREB phosphorylation in both control and stress groups. These results contrast with other findings showing that acute nicotine treatments did not alter phosphoCREB levels in the NAc (Brunzell et al., 2003; Pandey et al., 2001; Pluzarev and Pandey, 2004). However, in these studies they used a higher dose of nicotine (2.0 mg/kg) (Pandey et al., 2001; Pluzarev and Pandey, 2004) a different specie (mouse), and route of administration (oral) (Brunzell et al., 2003) when compared to our study.

Evidence shows that ERK can be activated by phosphoCREB (Nakayama et al., 2001; Ying et al., 2002). Since we found decreased CREB activation in the NAc, we could expect that phosphoERK levels would also be reduced in this brain area. However, our results did not show changes in phosphorylated ERK following exposure to variable stress or acute nicotine. Indeed ERK activity can be regulated by other pathways such as cAMP-dependent protein kinase (PKA) and calcium/calmodulin-dependent kinases (CaMKs) (Sgambato et al., 1998; Thomas and Huganir, 2004; Xing et al., 1996). Future studies should address this issue.

In summary, the current results show that exposure to variable stress induces long-lasting behavioral sensitization and increases the motivation to nicotine intake corroborating clinical findings showing the relationship between stress exposure and tobacco addiction.

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