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Restraint stress attenuates nicotine's locomotor stimulant but not discriminative stimulus effects in rats

Andrew C. Harris^{a,b}, Christina Mattson^a, David Shelley^{a,1}, and Mark G. LeSage^{a,b}

^aMinneapolis Medical Research Foundation, Minneapolis, MN

^bDepartment of Medicine, University of Minnesota Medical School, Minneapolis, MN

Abstract

Stress enhances the locomotor stimulant and discriminative stimulus effects of several addictive drugs (e.g., morphine) in rodents, yet interactions between stress and nicotine's effects in these behavioral models have not been well established. To this end, the current studies examined the effects of restraint stress on nicotine-induced locomotor activity and nicotine discrimination in rats. We used a novel approach in which onset of stress and nicotine administration occurred concurrently (i.e., simultaneous exposure) to simulate effects of stress on ongoing tobacco use, as well as a more traditional approach in which a delay was imposed between stress and nicotine administration (i.e., sequential exposure). Simultaneous exposure to stress reduced the rate of locomotor sensitization induced by daily injections of nicotine (0.4 mg/kg, s.c.). A lower dose of nicotine (0.1 mg/kg, s.c.) produced modest effects on activity that were generally unaffected by simultaneous exposure to stress. Sequential exposure to stress and nicotine (0.4 mg/kg, s.c.) slightly suppressed nicotine-induced activity, but did not influence rate of locomotor sensitization. Neither simultaneous nor sequential exposure to stress influenced the discriminative stimulus effects of nicotine (0.01 – 0.2 mg/kg, s.c.). These data show that restraint stress reduces nicotine's locomotor stimulant effects, particularly when onset of stress and nicotine exposure occurs simultaneously, but does not influence nicotine discrimination. These findings contrast with the ability of stress to enhance the effects of other drugs in these models. This study also suggests that studying the influence of simultaneous stress exposure on drug effects may be useful for understanding the role of stress in addiction.

Keywords

Nicotine; Addiction; Stress; Locomotor sensitization; Drug discrimination; Rat

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Corresponding Author: Andrew C. Harris, Ph.D., Minneapolis Medical Research Foundation, 701 Park Avenue, Minneapolis, MN 55415, harr0547@umn.edu, Telephone: 612-250-0863, Fax: 612-337-7372.

¹Now at the Fred Hutchinson Cancer Research Center, Seattle, WA

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1. Introduction

Stress contributes to addiction to nicotine and other drugs (Cleck and Blendy, 2008; Goeders, 2003; Kassel et al., 2003; Koob, 2013). For example, stressful life events increase drug consumption and are a common cause of relapse (e.g., Niaura et al., 2002; Sinha, 2009; Sinha et al., 2011). Elucidating the behavioral and neurobiological mechanisms mediating the relationship between stress and addictive drugs could lead to more effective prevention and treatment of drug addiction.

Preclinical models have been useful for understanding the role of stress in drug addiction. It is well established that stressors (e.g., restraint, food restriction) increase the locomotor stimulant effects of single or repeated injections of addictive drugs such as amphetamine, cocaine, and morphine (e.g., Ahmed et al., 1995; Antelman et al., 1980; Deroche et al., 1993; Shaham et al., 1995). Stress can also increase the discriminative stimulus (interoceptive) effects of certain drugs (e.g., cocaine) and/or produce drug-like discriminative stimulus effects itself (Fowler et al., 1993; Kohut et al., 2012; Mantsch and Goeders, 1998; Miczek et al., 1999). These effects may have relevance to the facilitation of drug addiction by stress (e.g., Lu et al., 2003; Marinelli and Piazza, 2002).

Effects of stress on nicotine's locomotor stimulant and discriminative stimulus effects have not been well established. Across different studies, restraint or other stressors enhanced, inhibited, or had no effect on nicotine-induced locomotor activity (e.g., Cadoni et al., 2003; Cruz et al., 2008; Kita et al., 1999; Leao et al., 2012; McCormick and Ibrahim, 2007). It is unclear which of the many methodological factors that differed across studies (e.g., nature of stressor, age and sex of the animals, nicotine dosing regimen) account for these mixed findings. Regardless, the inability of stress to consistently enhance nicotine's locomotor stimulant effects suggests that the relationship between stress and nicotine may be unique. No studies have examined effects of stress in a model of nicotine discrimination.

In most studies examining effects of stress on the locomotor stimulant or discriminative stimulus effects of drugs, a delay ranging from minutes to days is imposed between offset of stress and administration of drug / behavioral testing. While this approach has been very valuable, humans may also be exposed to stress and drugs simultaneously rather than sequentially (e.g., smoking in the presence of social stress). Models involving the use of simultaneous exposure to stress and drugs may therefore provide additional insights into the role of stress in addiction (see Zago et al., 2012). In addition, duration of the interval between stress and drug administration can influence magnitude of stress effects on drug-induced locomotor stimulation (e.g., Stohr et al., 1999; Vanderschuren et al., 1997). As such, the parameters of contiguity between stress and drug exposure may represent an important variable in these models.

The current studies examined the effects of restraint stress on the locomotor stimulant (Experiment 1) or discriminative stimulus (Experiment 2) effects of nicotine. Experiment 2 also examined the ability of stress to produce nicotine-like discriminative stimulus effects itself. Stress-nicotine interactions were examined using either a novel approach in which onset of stress and nicotine exposure occurred concurrently (i.e., simultaneous exposure), as

well as a more traditional approach in which a short delay was imposed between offset of stress exposure and nicotine administration (i.e., sequential exposure).

2. Materials and Methods

2.1. Animals

Male Holtzman Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 275-325 g at arrival were individually housed in temperature- and humidity-controlled colony rooms with unlimited access to water. Rats in Experiment 1 were housed under a regular 12-hr light/dark cycle and tested for locomotor activity during the light (inactive) phase. Rats in Experiment 2 were housed under a reversed 12-hr light/dark cycle so that discrimination testing would occur during the dark (active) phase. Locomotor activity and nicotine discrimination are typically tested during these phases of the light/dark cycle in our lab and several others (Bevins and Besheer, 2001; Forget et al., 2010; Harris et al., 2012; LeSage et al., 2012). We used the same lighting conditions in the present studies to examine how stress might affect these behavioral measures in our standard models. Beginning one week after arrival, all rats were foodrestricted to ≈ 18 g/day rat chow to maintain good health and to prevent rats from becoming too large to fit in the restraint bottles (described below). This mild degree of food restriction (approximately 90-95% of free access intake) does not itself represent a significant stressor (see Garcia-Belenguer et al., 1993; Heiderstadt et al., 2000). Protocols were approved by the Minneapolis Medical Research Foundation Animal Care and Use Committee and were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Drugs

Nicotine bitartrate (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile saline. The pH of all nicotine solutions was adjusted to 7.4 with dilute NaOH. Nicotine doses are expressed as the base. All injections were administered s.c. in a volume of 1.0 ml/kg.

2.3. Restraint stress

The stress condition involved immobilization of animals in eight glass restraint bottles (interior volume = 800 ml) attached to an 8-port cylinder (TSE systems, Bad Homburg, Germany) that provided nose-only exposure to fresh air (see Harris et al. (2010) for further details). For the no stress condition, animals remained undisturbed in their transport chambers rather than being exposed to restraint stress.

2.4. Nicotine discrimination

The general apparatus and training procedure used here have been described in detail elsewhere (LeSage et al., 2009). Briefly, animals ($N = 15$) were trained to discriminate nicotine (0.1 mg/kg) from saline using a 2-lever discrimination procedure. This training dose was used because it produces more clinically relevant nicotine serum levels and greater sensitivity to certain experimental manipulations than higher training doses (e.g., 0.4 mg/kg) (see Stolerman et al., 1984; Stolerman and Smith, 2009). Lever pressing was reinforced under a terminal variable interval 15-sec schedule using 45-mg food pellets. Discrimination was assessed twice weekly (Tues and Fri) during 2-min extinction test sessions.

Discrimination was considered stable when a) >80% responding occurred on the injection-appropriate lever during two consecutive saline and nicotine test sessions, b) >95% injection-appropriate responding occurred on six consecutive training sessions, and c) response rates (total responses/session) were stable (no trend across these four test sessions and six training sessions). Animals that acquired stable discrimination under these conditions (n = 10) were tested in Experiment 2a. For the remaining animals (n = 5), the nicotine training dose was increased from 0.1 to 0.2 mg/kg. All of these animals acquired stable discrimination with this nicotine dose and were tested in Experiment 2b.

2.5. Experimental protocols

2.5.1. Experiment 1a: Effects of simultaneous exposure to stress and nicotine on nicotine's locomotor stimulant effects—On each of two consecutive habituation days, rats (N = 56) were tested for locomotor activity in open field activity chambers (described in Cornish et al., 2011; Harris et al., 2010; Roiko et al., 2008) for 30 min (pre-test). Five min after the pre-test, rats were injected with s.c. saline and immediately exposed to either restraint stress (n = 30) or no stress (n = 26) for 10 min. Five min later, rats were again tested for activity for 30 min (post-test). Within each stress condition (stress or no stress), total distance traveled during the post-test on the second day of habituation was used to match animals into groups (see below) with similar baseline activity levels.

The test phase began two days after completion of habituation. On each test day, rats in the Sal + Stress group (negative control for stress condition, n = 11) continued to be treated as during habituation (i.e., 30 min pre-test, s.c. saline injection, 10 min restraint stress, 30 min post-test). The 0.1 Nic + Stress (n = 10) and 0.4 Nic + Stress (n = 9) groups were treated identically with the exception that rats were injected with 0.1 or 0.4 mg/kg nicotine immediately prior to stress exposure. Both of these nicotine doses have been shown to induce locomotor sensitization (Clarke and Kumar, 1983; Domino, 2001). The 0.4 mg/kg dose has also been used in several studies examining effects of stress on nicotine's locomotor stimulant effects (e.g., Cruz et al., 2008; Leao et al., 2012). The Sal + No Stress, 0.1 Nic + No Stress, and 0.4 Nic + No Stress groups (n = 8-10/group) were treated identically except that animals were not exposed to restraint stress. Rats were treated in this manner 5 days a week for 3 weeks (15 test days total). Drug administration and activity testing were then suspended for 10 days, after which all rats were tested as described above (challenge test).

2.5.2. Experiment 1b: Effects of sequential exposure to stress and nicotine on nicotine's locomotor stimulant effects—Two groups of rats (n = 8 each) were treated identically to the 0.4 Nic + Stress and 0.4 Nic + No Stress groups described above with the exception that rats were injected with 0.4 mg/kg nicotine immediately prior to the post-test (i.e., 5 min *after* exposure to stress/no stress).

2.5.3. Experiment 2a: Effects of simultaneous and sequential exposure to stress and nicotine on nicotine discrimination (0.1 mg/kg nicotine training dose)

2.5.3.1. General design: Rats underwent a total of 4 test phases (each preceded by a habituation phase) using a 2 (stress or no stress) x 2 (simultaneous or sequential exposure) within-subjects design, with the order of test phases counterbalanced across subjects.

2.5.3.2. Habituation phases: During habituation sessions, rats were injected with saline and immediately exposed to the same stress condition (stress or no stress) used in the subsequent test phase. Ten minutes after completion of stress/no stress exposure, rats were injected with saline or 0.1 mg/kg nicotine and immediately tested for discrimination as described above. This procedure was repeated twice per week subject to stable discrimination on intervening training days. Each test phase commenced when discrimination was stable under these conditions (same stability criteria as above).

2.5.3.3. Test phases: To examine effects of *simultaneous* exposure to stress and nicotine on nicotine discrimination, rats were administered nicotine (0, 0.01, 0.03, 0.05, 0.1, or 0.2 mg/kg) immediately prior to stress or no stress exposure (see Fig 1A). Ten min after completion of stress/no stress exposure (i.e., twenty min after nicotine injection), all animals were injected with saline and immediately tested for nicotine discrimination as above. The purpose of the saline injection prior to discrimination testing was to keep the total number of injections constant across the simultaneous and sequential exposure conditions (described below). Testing was repeated twice per week subject to stable discrimination on intervening training days, and nicotine doses were administered in a counterbalanced order across rats. A similar nicotine dose range has been used previously to generate nicotine generalization dose-effect functions (e.g., LeSage et al., 2012; LeSage et al., 2009; Philibin et al., 2005; Stolerman and White, 1996). To examine effects of *sequential* exposure to stress and nicotine on nicotine discrimination, the above protocol was repeated except that saline was administered immediately prior to stress/no stress exposure and nicotine (same dose range as above) was administered immediately prior to discrimination testing (See Fig 1B).

2.5.4. Experiment 2b: Effects of simultaneous and sequential exposure to stress and nicotine on nicotine discrimination (0.2 mg/kg nicotine training dose)—Rats were tested using the same experimental protocol as in Experiment 2a with the exception that the nicotine training dose was 0.2 mg/kg.

2.6. Statistical analyses

2.6.1. Locomotor activity—Locomotor activity was measured as total horizontal distance traveled (in cm) over each 30-min post-test. In Experiment 1a, data during habituation and test phases were analyzed using separate three-factor ANOVAs with stress condition and nicotine dose as between-subject factors and session as a within-subject factor. Following a significant three-way interaction, data for each nicotine dose were analyzed using separate two-factor (stress x session) ANOVAs followed by Bonferroni post hoc tests comparing stress conditions at each session. Data during the challenge test were

analyzed using two-factor ANOVA with stress condition and nicotine dose as between-subject factors.

To assess the rate of locomotor sensitization, defined as a significant increase in nicotine's locomotor stimulant effects across repeated exposures (DiFranza and Wellman, 2007), linear regression analyses were performed on each animal's data from sessions 1-15. In this analysis, higher slope coefficients indicate a greater rate of sensitization (see Badiani et al., 1995; Fraioli et al., 1999). Sensitization data, as well as locomotor data collapsed across sessions 11-15 (i.e., the sessions in which nicotine's effects were most apparent, see Fig 2), were analyzed using separate two-factor ANOVAs with stress condition and nicotine dose as between-subject factors, followed by Bonferroni post-hoc tests comparing stress conditions at each nicotine dose. Data within each stress condition were also analyzed using a one-factor ANOVA with nicotine dose as a factor, followed by Dunnett's post hoc tests comparing each nicotine dose to saline.

To evaluate whether collapsing data across each 30-min session obscured any effects, within-session activity during session 1 and sessions 11-15 were separated into 5-min blocks and analyzed using separate two-factor ANOVAs with stress condition and 5-min block as factors, followed by Bonferroni post hoc tests comparing stress conditions at each 5-min block.

Data for Experiment 1b were analyzed as described for Experiment 1a, except that rate of sensitization and overall activity levels during sessions 11-15 were analyzed using independent samples t-tests rather than ANOVA because only a single nicotine dose was studied.

2.6.2. Nicotine discrimination—Nicotine discrimination was measured as the percentage of responding on the nicotine-appropriate lever (%NLR) and overall response rate (responses/second) during the 2-min extinction test sessions. In Experiments 2a and 2b, data during the simultaneous and sequential stress assessments were analyzed using separate 2-factor ANOVAs with stress condition and nicotine dose as within-subject factors.

3. Results

3.1. Experiment 1a: Effects of simultaneous exposure to stress and nicotine on nicotine's locomotor stimulant effects

3.1.1. Habituation phase—There was a significant main effect of stress ($F(1,50) = 6.6$, $p < 0.05$), reflecting a suppression of activity in all groups exposed to restraint stress (see sessions -1 and 0 in Fig 2A-2C). There was also a main effect of session ($F(1, 50) = 4.7$, $p < 0.05$), but no effect of subsequent nicotine dose or interactions between stress, session, and subsequent nicotine dose (Fig 2A – 2C).

3.1.2. Test phase—An initial 3-way ANOVA indicated significant main effects of stress ($F(1, 50) = 6.8$, $p < 0.05$), nicotine dose ($F(2, 50) = 16.3$, $p < 0.0001$), and session ($F(14, 700) = 17.6$, $p < 0.0001$), a significant nicotine dose $\times$ session interaction ($F(28, 700) = 5.8$,

$p < 0.0001$), and a significant stress $\times$ nicotine dose $\times$ session interaction ($F(28, 700) = 3.3, p < 0.0001$).

Consistent with previous studies (e.g., Faraday, 2002), stress produced a transient suppression of activity in drug-naïve animals (Fig 2A). There was no effect of stress in saline-treated rats, but there was a significant effect of session ($F(14, 238) = 3.5, p < 0.0001$) and a significant stress $\times$ session interaction ($F(14, 238) = 4.1, p < 0.0001$). Activity in the Sal + Stress group was lower than in the Sal + No Stress group during session 1 (Bonferroni $t(255) = 5.1, p < 0.01$) but not during subsequent sessions (Fig 2A).

There was a marginally significant effect of stress for rats treated with 0.1 mg/kg nicotine ($F(1,16) = 4.3, p = 0.054$), reflecting a tendency for stress to suppress activity across all sessions (Fig 2B). There was also a significant effect of session ($F(14, 224) = 8.7, p < 0.0001$), but no stress $\times$ session interaction.

There was no effect of stress in rats administered 0.4 mg/kg nicotine, but there was an effect of session ($F(14, 238) = 12.7, p < 0.0001$) and a stress $\times$ session interaction ($F(14, 238) = 3.5, p < 0.0001$). Activity was consistently higher in the 0.4 Nic + No Stress group compared to the 0.4 Nic + Stress group throughout sessions 6-15 (Fig 2C), although this difference did not achieve significance during any individual session.

3.1.3. Challenge—Stress did not significantly affect activity for any nicotine dose during the challenge (Fig 2A-2C). There was a significant effect of nicotine dose ($F(2, 50) = 12.7, p < 0.0001$), but no effect of stress or nicotine dose $\times$ stress interaction.

3.1.4. Rate of sensitization—Comparison of slope coefficients for data across sessions 1-15 indicated a marginally significant effect of stress ($F(1, 50) = 3.1, p = 0.08$), a significant effect of nicotine dose ($F(2, 50) = 19.3, p < 0.0001$), and a significant stress $\times$ nicotine dose interaction ($F(2, 50) = 10.0, p < 0.001$). Slope coefficients were significantly reduced in the 0.4 Nic + Stress group compared to the 0.4 Nic + No Stress group ($t(50) = 4.5, p < 0.01$), but were not affected by stress in rats receiving 0.1 mg/kg nicotine (Fig 3A). Slope coefficients appeared higher in the Sal + Stress group compared to the Sal + No Stress group (Fig 3A), but this difference did not achieve significance ($t(50) = 1.8, p = 0.08$). There was a significant effect of nicotine dose ($F(2,23) = 19.5, p < 0.0001$) within the no stress condition, with higher slope coefficients at the 0.4 mg/kg (Dunnett $q(23) = 6.2, p < 0.0001$) and 0.1 mg/kg ($q(23) = 3.0, p < 0.05$) doses of nicotine compared to saline, but no significant effect of nicotine dose in the stress condition (Fig 3A).

3.1.5. Overall activity levels during sessions 11-15—There was a significant main effect of stress ($F(1, 50) = 6.2, p < 0.05$) and nicotine dose ($F(2, 50) = 16.5, p < 0.0001$), but no significant interaction. Stress significantly reduced activity in rats administered 0.4 mg/kg nicotine ($t(50) = 2.6, p < 0.05$), but not 0.1 mg/kg nicotine or saline, compared to unstressed animals (Fig 3B). There was a significant effect of nicotine dose for the no stress condition ($F(2,23) = 7.5, p < 0.01$), with activity significantly increased at 0.4 mg/kg nicotine ($q(23) = 3.8, p < 0.01$) but not 0.1 mg/kg nicotine ($q(23) = 1.7, p = 0.18$) compared to saline. There was also a significant effect of nicotine dose within the stress condition

($F(2,27)=11.8, p < 0.001$), with activity significantly increased at 0.4 mg/kg ($q(27) = 4.8, p < 0.01$) and 0.1 mg/kg ($q(27) = 2.5, p < 0.05$) compared to saline (Fig 3B).

3.1.6. Within-session activity levels during sessions 1 and 11-15—During session 1, there were significant effects of stress ($F(1, 17) = 7.3, p < 0.05$), 5-min block ($F(5, 85) = 32.3, p < 0.0001$), and a significant stress x 5-min block interaction ($F(5, 85) = 5.3, p < 0.001$) in saline-treated rats. Activity in the Sal + Stress group was suppressed compared to the Sal + No Stress group during the first 5-min block of session 1 ($t(102) = 5.5, p < 0.01$), but not during subsequent 5-min blocks (Fig 4A). There was a significant effect of 5-min block in rats receiving 0.1 mg/kg nicotine ($F(5, 80) = 11.2, p < 0.0001$) and 0.4 mg/kg nicotine ($F(5, 85) = 16.8, p < 0.001$) during session 1, but no effect of stress or stress x 5-min block interaction for either nicotine dose (Fig 4B and 4C).

Effects of restraint stress on nicotine-induced activity during sessions 11-15 (see Fig 3B) were most apparent toward the end of each 30 min session (Fig 4D-4F). There was no effect of stress in saline-treated rats, but there was a significant effect of 5-min block ($F(5, 85) = 93.6, p < 0.0001$) and a significant stress x 5-min block interaction ($F(5, 85) = 6.1, p < 0.0001$). Activity in the Sal + Stress group was modestly elevated compared to the Sal + No Stress group during the first 5-min block ($t(102) = 3.1, p < 0.05$), but groups did not differ during subsequent blocks (Fig 4D). There were significant effects of stress ($F(1, 16) = 5.2, p < 0.05$), 5-min block ($F(5, 80) = 57.6, p < 0.0001$) and a stress x 5-min block interaction ($F(5, 80) = 4.6, p < 0.001$) in rats receiving 0.1 mg/kg nicotine, with activity in the Nic 0.1 + Stress group significantly suppressed during the final 10 min of the session ($t(96) = 3.1$ or $3.2, p < 0.05$ or 0.01 ; Fig 4E). There was no significant effect of stress in animals administered 0.4 mg/kg nicotine, but there was a significant effect of 5-min block ($F(5, 85) = 88.7, p < 0.0001$) and a significant stress x 5-min block interaction ($F(5, 85) = 9.7, p < 0.001$). Activity in the Nic 0.4 + Stress group was significantly suppressed compared to activity in the Nic 0.4 + No Stress group during the last 5-min block of the session ($t(102) = 3.3, p < 0.01$; Fig 4F).

3.2. Experiment 1B: Effects of sequential exposure to stress and nicotine on nicotine's locomotor stimulant effects

3.2.1. Habituation phase—There was a significant main effect of stress ($F(1, 14) = 14.1, p < 0.01$), reflecting a stress-induced suppression of activity during habituation (Fig 5A), but no effect of session or stress x session interaction.

3.2.2. Test phase—There was a significant effect of stress ($F(1, 14) = 5.5, p < 0.05$), reflecting an overall suppression of activity in the Stress + 0.4 Nic group (see Fig 5A), and a significant effect of session ($F(14, 196) = 22.8, p < 0.0001$). The stress x session interaction did not achieve significance ($F(14, 196) = 1.6, p = 0.09$). Activity in the Stress + 0.4 Nic group was significantly lower compared to the No Stress + Nic 0.4 group during sessions 5 and 7 ($t(210) = 3.2$ or $3.4, p < 0.01$).

3.2.3. Challenge—Stress did not significantly affect activity during the challenge (Fig 5A).

3.2.4. Rate of sensitization and activity levels during sessions 11-15—The No Stress + 0.4 Nic and Stress + 0.4 Nic groups did not differ in terms of rate of sensitization (Fig 5B) or either overall (Fig 5C) or within-session (data not shown) activity levels during sessions 11-15.

3.3. Experiment 2a: Effects of simultaneous and sequential exposure to stress and nicotine on nicotine discrimination (0.1 mg/kg nicotine training dose)

3.3.1. %NLR—Nicotine's discriminative stimulus effects were not influenced by either simultaneous or sequential exposure to stress and nicotine. There were significant effects of nicotine dose in both the simultaneous ($F(5, 45) = 62.4, p < 0.0001$) and sequential ($F(5, 45) = 104.8, p < 0.0001$) exposure conditions, reflecting increases in %NLR as the nicotine test dose increased (Fig 6A and 6B). However, there was no effect of stress or nicotine dose x stress interaction for either condition. The lack of difference between stress and no stress when saline was used as the test dose (see Fig 6A and 6B) indicates that restraint stress itself did not substitute for nicotine.

3.3.2. Response rates—There was no effect of nicotine dose, stress, or nicotine dose x stress interaction on response rates during the simultaneous stress condition (Table 1). There was an effect of nicotine dose during the sequential stress condition ($F(5, 45) = 4.2, p < 0.01$), reflecting a suppression of response rates following administration of 0.2 mg/kg nicotine in both the presence and absence of stress (Table 1), but no effect of stress or nicotine dose x stress interaction.

3.4. Experiment 2b: Effects of simultaneous and sequential exposure to stress and nicotine on nicotine discrimination (0.2 mg/kg nicotine training dose)

3.4.1. %NLR and response rates—Nicotine discrimination was not influenced by stress in rats trained with a 0.2 mg/kg dose of nicotine. There were significant effects of nicotine dose for both the simultaneous stress condition ($F(5, 20) = 28.3, p < 0.0001$) and the sequential stress condition ($F(5, 20) = 23.3, p < 0.0001$) (Fig 6C and 6D), but no effect of stress or nicotine dose x stress interaction for either condition. There was also no effect of nicotine dose, stress, or nicotine dose x stress interaction on response rates for either the simultaneous or sequential stress conditions (data not shown).

4. Discussion

The main findings of these studies were that 1) simultaneous exposure to restraint stress reduced the rate of sensitization induced by repeated injections of nicotine (0.4 mg/kg; Experiment 1a); 2) nicotine (0.1 mg/kg) produced modest effects on activity that were not markedly affected by simultaneous stress (Experiment 1a); 3) sequential exposure to stress and nicotine (0.4 mg/kg) modestly suppressed nicotine's locomotor effects throughout the protocol, but did not influence rate of sensitization (within-subject increase in nicotine's effects across repeated exposures) *per se* (Experiment 1b); 4) neither simultaneous nor sequential exposure to stress and nicotine (0.01 – 0.2 mg/kg) influenced nicotine's discriminative stimulus effects in rats trained to discriminate nicotine (0.1 or 0.2 mg/kg)

from saline (Experiment 2a and 2b). Restraint stress also failed to produce nicotine-like discriminative stimulus effects itself in Experiments 2a and 2b.

The present findings complement a report that social stress reduced the locomotor stimulant effects of repeated nicotine injections in adolescent rats (McCormick and Ibrahim, 2007). However, other studies found that restraint or other stressors (e.g., food restriction) either enhanced (Kita et al., 1999; Leao et al., 2012; McCormick et al., 2004) or had no effect (Cadoni et al., 2003; Cruz et al., 2008; Faraday et al., 2005; McCormick et al., 2005) on nicotine's locomotor effects. Reconciling inconsistencies in the literature regarding the influence of stress on nicotine's locomotor effects was beyond the scope of our study. In fact, it is unlikely that any single study could fully address this issue, as the relevant studies have differed in terms of several potentially important methodological variables (e.g., nicotine dosing regimen, type and frequency of stressor, rat strain, etc). Nonetheless, our data identify a variable (i.e., simultaneous versus sequential stress exposure) that could be included in a comprehensive series of studies evaluating the critical methodological factors influencing the relationship between stress and nicotine's locomotor effects.

A limitation of our study, as well as most others in this area (e.g., Cruz et al., 2008; Kita et al., 1999; Leao et al., 2012), is that other drugs of abuse (e.g., cocaine) were not tested. As such, comparison of our data with previously reported interactions between stress and other drugs should be approached with caution. Notwithstanding this important caveat, our data contrast with the ability of stress to enhance the locomotor stimulant and discriminative stimulus effects of drugs including amphetamine and cocaine (e.g., Deroche et al., 1993; Kohut et al., 2012). Similarly, stress enhances the potential for several drugs (e.g., cocaine) to increase the reinforcing effects of electrical brain stimulation, an effect not observed with nicotine (Cabeza de Vaca and Carr, 1998). In contrast, stress produces similar effects for nicotine as for other drugs in other behavioral assays (e.g., reinstatement of extinguished i.v. self-administration, Buczek et al., 1999; Zislis et al., 2007). Further characterization of the common and unique interactions between stress and the effects of nicotine versus other drugs could provide insights into the relative clinical potential of pharmacotherapies targeting stress systems (e.g., corticotropin-releasing factor antagonists) for treating different forms of drug abuse.

Restraint or social defeat stress can itself produce similar discriminative stimulus effects as cocaine or amphetamine (Mantsch and Goeders, 1998; Miczek et al., 1999). The inability of restraint stress to substitute for nicotine in this study may reflect the fact that the current nicotine training doses (0.1 or 0.2 mg/kg) were considerably lower than those shown to produce anxiogenic effects in rats (0.4-0.6 mg/kg, see Cheeta et al., 2001; File et al., 1998). The use of higher, anxiogenic nicotine doses for discrimination training could potentially lead to crossgeneralization with stress.

Comparison of data across Experiments 1a and 1b indicates that stress more effectively inhibited nicotine's locomotor effects when stress and nicotine were administered simultaneously rather than sequentially. In the only other study to evaluate the effects of simultaneous stress exposure on drug-induced locomotor activity, a history of repeated exposure to nicotine and simultaneous restraint stress enhanced nicotine's acute locomotor

stimulant effects in adolescent but not adult rats (Zago et al., 2012). Effects of sequential exposure to stress and nicotine were not studied. It is difficult to compare these findings with ours given the substantial methodological differences across studies (e.g., nicotine's locomotor effects in Zago et al. (2012) were only measured 3 days after completion of the nicotine/stress regimen). Nonetheless, together these studies support the utility of using simultaneous stress procedures to understand the role of stress in drug addiction.

Although outside the scope of this study, elucidating the mechanisms underlying the inhibition of nicotine's locomotor stimulant effects by stress represents an important area for future work. One potential mediator is the stress hormone corticosterone. Corticosterone can inhibit function of nicotinic acetylcholine receptors (Caggiula et al., 1998; Ke and Lukas, 1996), nicotine's primary site of action, and can also attenuate a number of nicotine's behavioral and physiological effects (e.g., analgesia, bradycardia, see Caggiula et al., 1993; Pauly et al., 1990; Pauly et al., 1988). Moreover, restraint stress-induced corticosterone release attenuates the ability of nicotine to stimulate the mesolimbic dopamine system (Enrico et al., 2013), a critical neurobiological mechanism mediating locomotor sensitization (DiFranza and Wellman, 2007). Nonetheless, not all findings predict an inhibition of nicotine's locomotor effects by corticosterone (see Johnson et al., 1995). Measurement of corticosterone and/or evaluation of the effects of manipulating corticosterone levels (e.g., via adrenalectomy) in the current locomotor models is needed to address the role of corticosterone in our findings. Alternatively, other mechanisms such as effects of stress on nicotine pharmacokinetics (Winders et al., 1988) and/or its effects on neurotransmitter systems implicated in nicotine's locomotor stimulant effects (e.g., serotonin, Zaniewska et al., 2010) may also account for our findings.

Stress inhibited nicotine's locomotor stimulant but not discriminative stimulant effects in this study. These data complement previous reports that different mechanisms can mediate nicotine's effects in these behavioral models. For example, while many studies support an important role for dopamine in nicotine's locomotor effects (e.g., Clarke, 1990; Mao and McGehee, 2010; Vezina et al., 2007), dopamine's involvement in nicotine discrimination has been less consistent (Corrigall and Coen, 1994; Smith and Stolerman, 2009). This dissociation may be relevant to the current findings, as a stress-induced inhibition of nicotine's effects on mesolimbic dopamine release (Enrico et al., 2013) could potentially inhibit nicotine's locomotor effects without influencing its discriminative stimulus effects.

Alternatively, the different effects of stress in Experiments 1 and 2 may reflect methodological factors that differed across studies other than behavioral measure. For example, the nicotine dose on which stress produced its greatest effects in Experiment 1 (0.4 mg/kg) was not tested in Experiment 2. Stress may have therefore influenced nicotine discrimination had a 0.4 mg/kg dose of nicotine been tested. Given that numerous behaviorally relevant hormones or neurotransmitters exhibit circadian rhythmicity (e.g., corticosterone, dopamine, see Hamilton et al., 2013; McClung, 2007), the fact that animals were tested during different phases of the light/dark cycle in Experiments 1 and 2 could also account for the different effects of stress across studies.

In conclusion, the current study demonstrated that restraint stress reduced nicotine's locomotor stimulant effects without influencing its discriminative stimulus effects. Our findings also suggest that the use of simultaneous exposure to stress and drug may represent an important variable in studying stress-drug interactions. Further development of these models, including incorporation of more ethologically relevant stressors (e.g., social stress) and extension to other behavioral models (e.g., conditioned place preference), may be useful for understanding the role of stress in nicotine addiction.

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Highlights

1. We studied effects of stress on nicotine's locomotor and interoceptive effects in rats.
2. Stress and nicotine were administered simultaneously or sequentially.
3. Simultaneous but not sequential stress markedly reduced nicotine's locomotor effects.
4. Neither simultaneous nor sequential stress exposure affected nicotine discrimination.
5. Simultaneous stress exposure reduced nicotine's locomotor but not interoceptive effects.

Figure 1. Procedural timeline for evaluating effects of simultaneous (A) and sequential (B) exposure to stress and nicotine on nicotine discrimination in Experiment 2. See text for further details.

Figure 2.

Total distance traveled (Mean $\pm$ SEM) during post-exposure tests during habituation, test, and challenge phases in animals administered saline (A), 0.1 mg/kg nicotine (B), or 0.4 mg/kg nicotine (C) and tested under Stress (dark symbols) or No Stress (light symbols) conditions in Experiment 1a. C = challenge test. **Significantly different from No Stress condition at that session, $p < 0.01$.

Figure 3.

(**A**) Slope coefficients (Mean $\pm$ SEM) during sessions 1-15 and (**B**) total distance traveled (Mean $\pm$ SEM) during sessions 11-15 in Experiment 1a. # Significantly different from No Stress condition at that dose, $p < 0.05$ or 0.01 . *, ** Significantly different from saline (0 mg/kg) for that stress condition, $p < 0.05$ or 0.01 .

Figure 4.

Total distance traveled (Mean $\pm$ SEM) during each 5-min block during session 1 in animals administered saline (A), 0.1 mg/kg nicotine (B), or 0.4 mg/kg nicotine (C) and tested under Stress (dark symbols) or No Stress (light symbols) conditions in Experiment 1a. Data from sessions 11-15 are shown in (D), (E), and (F). *,** Significantly different from No Stress condition at that 5-min block, $p < 0.05$ or 0.01 .

Figure 5.

(A) Distance traveled (Mean $\pm$ SEM) during post-exposure tests during habituation, sensitization, and challenge phases in animals exposed to Stress (dark symbols) or No Stress (light symbols) prior to 0.4 mg/kg nicotine in Experiment 1b. *Significantly different from No Stress condition (main effect), $p < 0.05$. #Significantly different from No Stress condition at that session, $p < 0.05$ or 0.01 . (C) Slope coefficients (Mean $\pm$ SEM) during sessions 1-15 and (B) total distance traveled (Mean $\pm$ SEM) during sessions 11-15 in Experiment 1b.

0.1 mg/kg training dose

0.2 mg/kg training dose

Figure 6.

Percent of total responses on the nicotine-appropriate lever (mean $\pm$ SEM) following s.c. injection of saline or nicotine (0.01-0.2 mg/kg) in the presence or absence of simultaneous or sequential stress in Experiment 2a (0.1 mg/kg nicotine training dose, A and B) and Experiment 2b (0.2 mg/kg nicotine training dose, C and D).

Overall response rate (total responses/sec, Mean $\pm$ SEM) in rats trained with a 0.1 mg/kg training dose and injected with nicotine (0.0-0.2 mg/kg) in either the presence or absence of simultaneous (top) or sequential (bottom) exposure to stress.

Table 1

	Nicotine (mg/kg)					
	0	0.01	0.03	0.05	0.1	0.2
<u>Simultaneous</u>						
No Stress	0.61 $\pm$ 0.10	0.58 $\pm$ 0.09	0.58 $\pm$ 0.09	0.56 $\pm$ 0.12	0.55 $\pm$ 0.09	0.57 $\pm$ 0.08
Stress	0.56 $\pm$ 0.12	0.70 $\pm$ 0.10	0.66 $\pm$ 0.10	0.51 $\pm$ 0.10	0.61 $\pm$ 0.12	0.42 $\pm$ 0.06
<u>Sequential</u>						
No Stress	0.58 $\pm$ 0.11	0.52 $\pm$ 0.13	0.45 $\pm$ 0.10	0.52 $\pm$ 0.11	0.52 $\pm$ 0.10	0.36 $\pm$ 0.07
Stress	0.65 $\pm$ 0.11	0.55 $\pm$ 0.14	0.54 $\pm$ 0.12	0.51 $\pm$ 0.08	0.51 $\pm$ 0.09	0.26 $\pm$ 0.06