Psychopharmacology (2012) 219:73–82
DOI 10.1007/s00213-011-2378-1

ORIGINAL INVESTIGATION

Effects of acute stress on acquisition of nicotine conditioned place preference in adolescent rats: a role for corticotropin-releasing factor 1 receptors

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Received: 12 September 2010 / Accepted: 7 June 2011 / Published online: 1 July 2011

Abstract
Rationale Studies indicate that adolescence is a time of increased sensitivity to the rewarding effects of nicotine, and that stress is associated with an increased risk for smoking initiation in this age group. It is possible that stress leads to increased nicotine use in adolescence by augmenting its rewarding properties. Corticotropin-releasing factor type 1 receptors (CRF-R1) mediate physiological and behavioral stress responses. They may also mediate stress-induced potentiation of activity in multiple neural substrates implicated in nicotine reward.
Objectives The aim of the present study was to determine the effect of acute stressor exposure on single trial nicotine conditioned place preference (CPP) in adolescent male rats using a biased CPP procedure and the role of CRF-R1 in this effect.
Results A single episode of intermittent footshock administered 24 h before the start of place conditioning dose-dependently facilitated acquisition of CPP to nicotine (0.2, 0.4, and 0.6 mg/kg). Pretreatment with CP-154,526 (20 mg/kg), a selective CRF-R1 antagonist, 30 min before footshock exposure significantly attenuated the effect of prior stress to facilitate nicotine CPP acquisition. CP-154,526 pretreatment had no effect in animals conditioned with a nicotine dose that produced CPP under non-stress conditions, suggesting a specific role for CRF-R1 following stress.
Conclusions Taken together, the results suggest that during adolescence, nicotine reward is enhanced by recent stressor exposure in a manner that involves signaling at CRF-R1. Information from studies such as this may be used to inform efforts to prevent and treat adolescent nicotine dependence.

Keywords Nicotine · Adolescence · Stress · Reward · Conditioned place preference · Corticotropin-releasing factor · CP-154,526

This research was supported by a grant from the Virginia Foundation for Healthy Youth to R.F. Smith and a grant from the American Psychological Association to J. Brielmaier. CP-154,526 was generously donated by Pfizer. The authors certify that they have no actual or potential conflicts of interest in relation to this article, nor do they have a financial relationship with either of the organizations that sponsored the research. The authors have full control of all primary data and agree to allow the journal to review the data if requested. The authors thank Drs. Theodore Dumas and Katherine McKnight for their valuable advice.

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Introduction

Smoking remains the leading preventable cause of death in the United States (National Center for Health Statistics 2004). The majority of all adult cigarette smokers began in adolescence (Centers for Disease Control 2008), suggesting that initiating tobacco use at an early age confers a higher risk for dependence (Kandel and Chen 2000). Rodent studies from our laboratory and others suggest that adolescents are more sensitive than adults to the rewarding effects of nicotine, which may serve to increase vulnerability to nicotine addiction (Belluzzi et al. 2004; Brielmaier et al. 2007; Shram and Lê 2010; Torres et al. 2008). Feelings of

reward and positive affect upon first exposure to tobacco strongly predict later dependence among adolescents (DiFranza et al. 2007; Kandel et al. 2007). However, little is known about factors that influence initial sensitivity to nicotine reward during this period. Stress has been shown to increase the rate of smoking initiation in adolescents (Koval et al. 2000; Rao et al. 2009), and rodent studies indicate that adolescence is a time of increased physiological (Goldman et al. 1973; Romeo et al. 2004a, b; Vazquez and Akil 1993) and behavioral (Spear 2000; Stone and Quartermain 1997) responsiveness to stressors. It is possible that stress serves to enhance nicotine's initial rewarding effects in adolescents, which could have lasting implications for the development of dependence.

Acute stress enhances the rewarding effects of several addictive drugs in rodents as measured using conditioned place preference (CPP) (Capriles and Cancela 1999; Dai et al. 2006; Der-Avakian et al. 2007; Grakalic et al. 2006; Matsuzawa et al. 1998a, b; Will et al. 1998). The mechanisms by which this occurs have not been fully elucidated. Current evidence points to stressor-induced potentiation of dopamine (DA) release within the mesolimbic pathway (Imperato et al. 1992; Kalivas and Duffy 1995), a critical mediator of the rewarding effects of nicotine and other abused drugs (Di Chiara and Imperato 1988; Laviolette et al. 2008; Sellings et al. 2008; Spina et al. 2006). Acute stressors both potentiate excitatory synaptic input and reduce inhibitory input onto ventral tegmental area (VTA) dopamine (DA) neurons (Niehaus et al. 2010; Saal et al. 2003) and perhaps accordingly increase the firing rates of these neurons (Anstrom and Woodward 2005). Moreover, a variety of acute stressors increase extracellular nucleus accumbens (NAc) DA levels (Imperato et al. 1992; Kalivas and Duffy 1995; Puglisi-Allegra et al. 1991), providing further evidence for stressor-induced potentiation of mesolimbic DA transmission. Some of these effects have been shown to last for at least 24 h (Anstrom and Woodward 2005; Niehaus et al. 2010; Saal et al. 2003), suggesting that acute stress may enhance nicotine reward via a long-lasting potentiation of activity in nicotine reward substrates.

Corticotropin-releasing factor (CRF) has emerged as a potential key mediator of the effects of stressors on drug responses. CRF is a peptide released from the paraventricular nucleus of the hypothalamus (PVN) during the hypothalamic-pituitary-adrenal (HPA) axis stress response (Smith and Vale 2006). CRF-like immunoreactivity is also found in "stress-sensitive" extrahypothalamic areas that project to mesolimbic structures as well as within the VTA and NAc themselves (Merchenthaler et al. 1982; Rodaros et al. 2007; Sawchenko et al. 1993; Swanson et al. 1983). CRF binds to two main receptor subtypes, CRF-R1 and CRF-R2 (Chalmers et al. 1996), as well as CRF-binding protein (CRF-BP), which inactivates CRF (Behan et al. 1995). CRF-R1 is the main receptor subtype found in pituitary corticotropes that release adrenocorticotropic hormone (Chalmers et al. 1996; Potter et al. 1994), and CRF-R1 signaling in extrahypothalamic areas is thought to be critical for behavioral stress responses (Koob and Heinrichs 1999). CRF-R1 is expressed in the VTA and NAc (Sauvage and Steckler 2001; Van Pett et al. 2000), and recent evidence suggests that it contributes to stressor-induced activation of mesolimbic DA release. Intermittent footshock causes release of CRF into the VTA (Wang et al. 2005), and CRF-induced increases in VTA DA neuron firing are abolished by a CRF-R1 (but not -R2) antagonist (Wanat et al. 2008). Additionally, the ability of CRF to enhance firing of VTA DA neurons is absent in CRF-R1 (but not -R2)-deficient mice (Wanat et al. 2008). The above findings suggest that release of CRF from the PVN and/or extrahypothalamic areas, and subsequent activation of CRF-R1, could mediate stressor-induced potentiation of mesolimbic DA activation and thus enhancement of drug reward. Support for this comes from a recent report that pretreatment with a CRF-R1 antagonist blocks stress-induced enhancement of cocaine CPP (Kreibich et al. 2009).

Given evidence for increased sensitivity to stress and nicotine reward during adolescence, we hypothesized that acute stress would enhance the rewarding effects of nicotine during this period. To test this, adolescent rats were exposed to a single episode of intermittent footshock and their subsequent CPP response to nicotine observed. Given the importance of the first tobacco experience in the development of dependence during adolescence, single-trial place conditioning (Brielmaier et al. 2007) was used to model initial sensitivity to nicotine reward. We further hypothesized that the effects of stress on nicotine reward are at least partly mediated by CRF-R1 signaling. To determine this, rats were pretreated with a selective CRF-R1 antagonist or vehicle prior to footshock exposure. Taken together, the results suggest that recent exposure to a stressor may augment the rewarding effects of nicotine through a mechanism involving CRF-R1.

Materials and methods

Subjects

Subjects were non-littermate adolescent male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA). Adolescence in the rat is defined as the age range between postnatal days 28 and 42 (Spear 2000); thus, the age of the animals tested here corresponds to early adolescence. Animals arrived upon weaning at postnatal day (P)21 and were housed in groups of 4–6 in clear Plexiglas cages with food and water

available ad libitum. The animals were maintained in a climate-controlled colony room at 21±2°C and a 12-h light–dark cycle, and all experiments were conducted during the light phase (0900–1800 h). A total of 371 rats were used in the following experiments. All experiments were completed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (National Research Council 1996) and the George Mason University Institutional Animal Care and Use Committee.

Drugs

(−) – Nicotine hydrogen tartrate (Sigma Chemical Company, St. Louis, MO) was dissolved in 0.9% saline and pH adjusted to 7.4. CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethyl-phenyl)-7H-pyrrolo[2,3-*d*]pyrimidin-4-*yl*]-ethylamine), a selective CRF-R1 antagonist generously donated by Pfizer (Groton, CT), was dissolved in a vehicle solution of 5% ethanol, 5% Cremophor EL (Sigma), and 90% saline (0.9%). Drugs were administered at an injection volume of 1 mL/kg body weight. Nicotine was administered subcutaneously (s.c.), and CP-154,526 was administered intraperitoneally (i.p.). The nicotine doses used, expressed as the free base, were chosen based on previous studies in which multiple conditioning trials produced nicotine CPP in adolescent rats (e.g., Thiel et al. 2009; Torres et al. 2008). The dose of CP-154,526 was chosen based on studies showing that this dose is sufficient to attenuate physiological and behavioral responses to stress in rats (e.g., Hikichi et al. 2000; Schulz et al. 1996).

Apparatus

Conditioned place preference

CPP testing was carried out in Plexiglas 2-sided conditioning boxes (Med Associates, VT) located in a very dimly lit (4–6 lx) testing room. Each side measured 21×42×30 cm. One side consisted of black walls with a stainless steel mesh floor and black paper lining. The other side consisted of white walls with a stainless steel rod floor and white paper lining. A black removable guillotine door was inserted during conditioning sessions to restrict the rats to their designated conditioning side. A camera mounted above the inserts recorded each trial, and information was sent to a computer in an adjacent room through Videotrack software. Between sessions on all experimental days, both chambers of the apparatus were cleaned with 70% ethanol, and paper lining was changed to remove odor cues.

Stressor

Footshock was administered in a brightly lit room separate from the CPP testing room. Rats received footshock in chambers made of Plexiglas and stainless steel measuring 30.5×25.4×30.5 cm, each equipped with two lights and enclosed within sound attenuation chambers (Habitest, Coulbourn Instruments). The floor of each chamber consisted of 18 stainless steel bars through which a shock generator (Coulbourn) delivered inescapable electric footshock. The schedule for intermittent footshock was programmed using FreezeScan software (Clever Sys Inc., Reston, VA) loaded onto a PC. Cameras mounted on the back wall of each chamber captured video during the sessions, and freezing behavior was quantified using the FreezeScan software. In order to make the shock chambers maximally distinguishable from the CPP apparatus, visual cues (black circles) were placed on the walls of the shock chambers and both chamber lights were on. The shock chambers were cleaned with 70% ethanol in between sessions to remove odor cues.

Procedures

Experiment 1: effects of footshock 24 h prior to conditioning on acquisition of nicotine CPP

The procedure for Experiment 1 is depicted in Table 1. On testing day 1 (P27), a 15-min pretest was conducted to determine initial side preference. Following 20 min of acclimation to the testing room, all animals (n=167) were placed in the CPP apparatus with the guillotine door removed. Placement was counterbalanced such that half the animals started in the white side and the other half in the black side. As we previously found (Brielmaier et al. 2007, 2008), the CPP apparatus was biased, with animals spending significantly less than half of the pretest (315.18± 3.16 s) in the white side (one sample t test, [$t(369)=-42.64$, $p<0.0001$]). Time spent in the non-preferred (white) side over the pretest was scored by an observer blind to experimental conditions. An animal was considered in a side when all four of its paws were situated there.

Following the pretest, animals were randomly assigned to receive either intermittent footshock (Stress groups) or no shock (No Stress groups). On testing day 2 (P28), animals in the Stress groups were transported from their home cages and received 10 min of intermittent footshock (0.8 mA intensity, 1 s pulse duration, mean intershock interval 36.5 s, range 10–70 s). Immediately following the footshock session, animals were returned to their home cages. Animals in the No Stress groups remained in their home cages for the duration of testing day 2.

Following footshock or no shock exposure, animals were randomly assigned to dose groups receiving one of three nicotine doses (0.2, 0.4, or 0.6 mg/kg, s.c.) or saline during conditioning. Single-trial nicotine place conditioning took place over testing days 3 and 4 (P29–30). Given the bias of

Table 1 Procedure for experiment 1

Day (age)	1 (P27)	2 (P28)	3 (P29)	4 (P30)	5 (P31)
	Pretest, 15 min	IFS, 10 min (controls: no shock)	Conditioning, 15 min N-NP (controls: S-NP) or S-P	Conditioning, 15 min N-NP (controls: S-NP) or S-P	Posttest, 15 min

IFS intermittent footshock, *N-NP (controls: S-NP)* conditioning with nicotine (0.2, 0.4, or 0.6 mg/kg, s.c.) or saline in the initially non-preferred side of the CPP apparatus, *S-P* conditioning with saline in the initially preferred side of the CPP apparatus

the apparatus, a biased procedure was used where nicotine was paired with the initially non-preferred (white) side of the apparatus. Order of nicotine conditioning (i.e., injection of nicotine and confinement to the non-preferred side on testing day 2 or 3) was counterbalanced within each treatment group. All conditioning sessions lasted 15 min, and each rat received one conditioning session per day.

On testing day 5 (P31), a 15-min, drug-free posttest was conducted to determine expression of nicotine CPP. Following acclimation to the testing room, animals were placed in the same side of the apparatus they started in for the pretest. As on the pretest day, the guillotine door was removed. Time spent in the white (non-preferred) side was again scored by an observer blind to experimental conditions. Following the conclusion of testing each day, animals were returned to their home cages.

Experiment 2: effect of CP-154,526 pretreatment on acquisition of nicotine CPP following footshock

The procedure for Experiment 2 (depicted in Table 2) was identical to that used for Experiment 1, except for the following. After the pretest on testing day 1, animals (n=152) were randomly assigned to both pretreatment and stress conditions. Peripherally administered CP-154,526 crosses the blood brain barrier and reaches maximal brain concentration after 20 min (Keller et al. 2002). Thus, on testing day 2, animals received a pretreatment injection of 20 mg/kg CP-154,526 (i.p.) (CP-154,526-Pretreated groups) or vehicle (Vehicle-Pretreated groups) 30 min before footshock or no shock and afterward returned to their home cages. Following footshock or no shock, animals within each stress condition were randomly assigned to drug groups receiving 0.4 mg/kg nicotine (s.c.) or saline during conditioning. This nicotine dose was chosen based on results from Experiment 1 (Fig. 1). Place conditioning sessions took place over testing days 3 and 4, and the posttest took place on testing day 5.

Experiment 3: effect of CP-154,526 pretreatment on acquisition of CPP under non-stress conditions

Based on the results from Experiment 1 (Fig. 1), the 0.4 mg/kg nicotine dose was chosen to determine the effects of CP-154,526 pretreatment in Experiment 2. Results from Experiment 2 (Fig. 2) showed that neither shocked nor non-shocked animals pretreated with CP-154,526 acquired CPP. In order to rule out the possibility that CP-154,526 pretreatment attenuates nicotine CPP independently of prior stressor exposure, we tested its effect when the highest nicotine dose, which produced significant CPP in non-shocked animals (Fig. 1), was used for conditioning. The procedure for Experiment 3 (depicted in Table 3) was identical to that used for Experiment 2, except for the following. After pretreatment with CP-154,526 (20 mg/kg, i.p.) or vehicle, all animals (n=51) remained in their home cages until conditioning sessions began on testing day 3. Animals in each drug group received either 0.6 mg/kg nicotine or saline (s.c.).

Statistical analysis

In all experiments, the dependent variable for measuring each rat's expression of CPP was a difference score

Table 2 Procedure for experiment 2

Day (age)	1 (P27)	2 (P28)	3 (P29)	4 (P30)	5 (P31)
	Pretest, 15 min	CP-154,526 (controls: Vehicle) ↓ 30 min IFS, 10 min (controls: no shock)	Conditioning, 15 min N-NP (controls: S-NP) or S-P	Conditioning, 15 min N-NP (controls: S-NP) or S-P	Posttest, 15 min

IFS intermittent footshock, *N-NP (controls: S-NP)* conditioning with nicotine (0.4 mg/kg, s.c.) or saline in the initially non-preferred side of the CPP apparatus, *S-P* conditioning with saline in the initially preferred side of the CPP apparatus

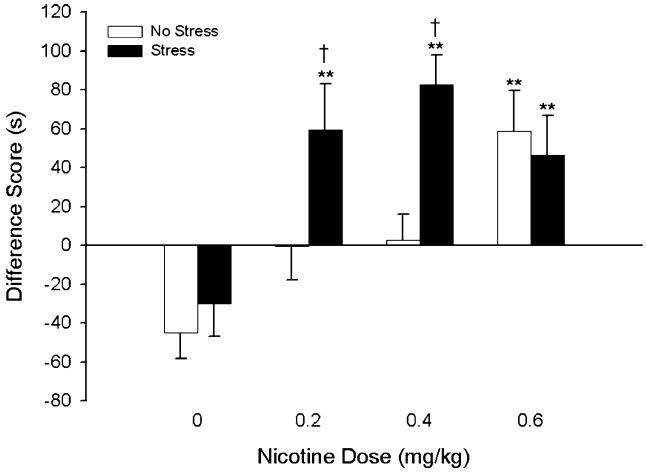

Fig. 1 Nicotine CPP in rats exposed to intermittent footshock or no shock 24 h prior to conditioning. *Asterisks* indicate significant difference from respective saline-conditioned group ($p \leq 0.005$). *Cross* indicates significant difference from respective non-shocked nicotine-conditioned group ($p < 0.05$). $n = 19–24$ per group

calculated as follows: time spent (seconds) in the nicotine-paired side of the apparatus during the posttest minus time spent there during the pretest. A positive score thus indicates increased preference for the drug-paired side, a negative score indicates aversion, and a score at or near zero indicates no change in preference. Difference scores were expressed as mean±SEM for each group. Data were analyzed using factorial analyses of variance (ANOVAs) with between-subjects factors of stress condition, nicotine dose (Experiments 1, 2, and 3) and pretreatment (Experiments 2 and 3). Simple effects analyses and one-way ANOVAs were conducted when the overall ANOVAs revealed significant interaction or main effects, respectively.

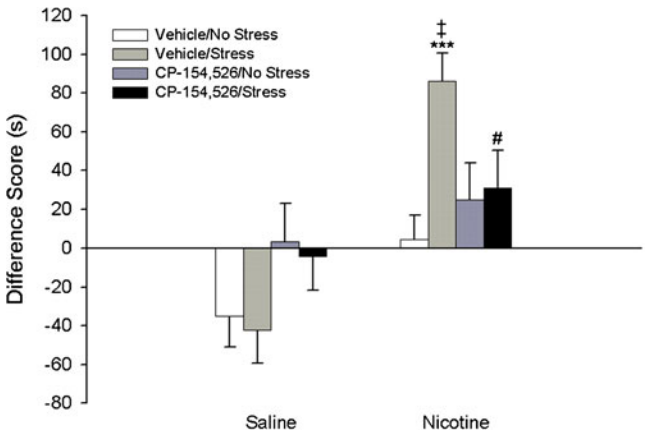

Fig. 2 Nicotine CPP in rats pretreated with CP-154,526 or vehicle 30 min prior to intermittent footshock or no shock. *Pound sign* indicates significant difference from Vehicle/Stress/Nicotine group ($p < 0.05$). *Asterisks* indicate significant difference from Vehicle/Stress/Saline group ($p < 0.0001$). *Double cross* indicates significant difference from Vehicle/No Stress/Nicotine group ($p = 0.001$). $n = 18–20$ per group

Individual post hoc comparisons between various treatment groups were also conducted based on a priori hypotheses using Fisher's PLSD test. In Experiment 2, freezing in response to footshock was quantified in light of evidence that CP-154,526 reduces behavioral responsiveness to stressors in rodents (Hikichi et al. 2000; Schulz et al. 1996). Freezing behavior in individual shocked animals was expressed as a percentage of total time spent freezing during the 10-min footshock session. Percent freezing was expressed as mean±SEM for each pretreatment group and data analyzed using an independent samples *t* test. The alpha level was set to 0.05 for all statistical tests.

Results

Experiment 1: effects of footshock 24 h prior to conditioning on acquisition of nicotine CPP

Exposure to intermittent footshock 24 h before conditioning facilitated acquisition of nicotine CPP. Animals in the Stress groups acquired nicotine CPP at all nicotine doses tested, but animals in the No Stress groups only acquired CPP to the highest nicotine dose (Fig. 1). The 2×4 (stress condition×dose) factorial ANOVA revealed significant main effects of stress condition [$F(1,159)=8.07$, $p=0.005$] and dose [$F(3,159)=11.01$, $p<0.0001$] and a significant stress condition×dose interaction [$F(3,159)=2.78$, $p<0.05$]. Post hoc tests revealed that at all three nicotine doses, shocked animals showed significant CPP compared to saline-conditioned animals exposed to footshock [$p \leq 0.005$ for each Stress/Nicotine group vs. the Stress/Saline group] and that non-shocked animals conditioned with 0.6 mg/kg nicotine showed significant CPP compared to non-shocked saline-conditioned animals [$p<0.0001$ for No Stress/0.6 mg/kg Nicotine vs. No Stress/Saline]. Post hoc tests also revealed a significant difference between the Stress and No Stress groups conditioned with 0.2 and 0.4 mg/kg nicotine, indicating significant CPP in shocked but not non-shocked animals [$p<0.05$ for Stress/0.2 mg/kg Nicotine vs. No Stress/0.2 mg/kg Nicotine and for Stress/0.4 mg/kg Nicotine vs. No Stress/0.4 mg/kg Nicotine].

Experiment 2: effect of CP-154,526 pretreatment on acquisition of nicotine CPP following footshock

Nicotine CPP

Footshock-induced facilitation of nicotine CPP acquisition was attenuated by pretreatment with CP-154,526 (Fig 2). The 2×2×2 (pretreatment×stress condition×drug) factorial ANOVA revealed a significant main effect of drug [$F(1,144)=20.39$, $p<0.0001$] as well as significant pretreat-

Table 3 Procedure for experiment 3

Day (age)	1 (P27)	2 (P28)	3 (P29)	4 (P30)	5 (P31)
	Pretest, 15 min	CP-154,526 (controls: Vehicle) ↓ 40 min Home cage until day 3	Conditioning, 15 min N-NP (controls: S-NP) or S-P	Conditioning, 15 min N-NP (controls: S-NP) or S-P	Posttest, 15 min

N-NP (controls: S-NP) conditioning with nicotine (0.6 mg/kg, s.c.) or saline in the initially non-preferred side of the CPP apparatus, *S-P* conditioning with saline in the initially preferred side of the CPP apparatus

ment×drug [$F(1,144)=4.87$, $p<0.05$] and stress condition×drug [$F(1, 144)=3.91$, $p=0.05$] interactions. Post hoc comparisons were made according to a priori predictions. Nicotine-conditioned animals pretreated with vehicle and exposed to intermittent footshock showed significant CPP relative to saline-conditioned controls [$p<0.0001$ for Vehicle/Stress/Nicotine vs. Vehicle/Stress/Saline] and relative to non-shocked nicotine-conditioned controls [$p=0.001$ for Vehicle/Stress/Nicotine vs. Vehicle/No Stress/Nicotine]. Nicotine-conditioned animals pretreated with CP-154,526 and exposed to intermittent footshock showed significantly attenuated CPP relative to the vehicle-pretreated group [$p<0.05$ for CP-154,526/Stress/Nicotine vs. Vehicle/Stress/Nicotine]. They also did not show significant CPP relative to stressed saline-conditioned controls or to non-shocked nicotine-conditioned controls [$p=0.16$ for CP-154,526/Stress/Nicotine vs. CP-154,526/Stress/Saline; $p=0.80$ for CP-154,526/Stress/Nicotine vs. CP-154,526/No Stress/Nicotine].

Footshock-induced freezing

The independent samples *t* test comparing percent freezing in CP-154,526 and vehicle-pretreated animals did not reveal significant differences between the two pretreatment groups [$t(75)=1.39$, $p=0.17$]. Percent freezing (mean±SEM) was 85.62%±0.92 for CP-154,526-pretreated animals and 83.43%±1.25 for vehicle-pretreated animals.

Experiment 3: effect of CP-154,526 pretreatment on acquisition of CPP under non-stress conditions

Non-shocked animals conditioned with 0.6 mg/kg nicotine acquired CPP that was unaffected by pretreatment with CP-154,526 (Fig. 3). The 2×2 (pretreatment×drug) factorial ANOVA revealed a significant main effect of drug [$F(1,47)=13.95$, $p<0.001$]. An independent samples *t* test revealed significantly greater difference scores in nicotine-conditioned animals relative to those conditioned with saline [$t(49)=3.59$, $p<0.001$], indicating significant CPP. No significant effects involving pretreatment were detected [$p>0.05$]. A planned comparison of difference scores from nicotine-conditioned animals pretreated with CP-154,526 and vehicle, respectively, revealed no difference between the two groups [$t(24)=0.67$, $p=0.51$ for CP-154,526/Nicotine vs. Vehicle/Nicotine].

Discussion

The present data provide evidence that a single exposure to a stressor enhances the subsequent rewarding effects of nicotine in adolescence and that CRF-R1 signaling is involved in this effect. In Experiment 1, animals exposed to intermittent footshock 24 h before the start of conditioning sessions acquired significant CPP at all nicotine doses tested, whereas animals not exposed to footshock only acquired CPP to the highest nicotine dose. Previous studies in adolescent rats have reported CPP to multiple doses within the dose range used here under non-stress conditions (e.g., Thiel et al. 2009; Torres et al. 2008). In contrast to the single nicotine conditioning trial used here, these studies used four nicotine conditioning trials. Results from Experiment 1 suggest that acute stress enhances the rewarding effects of lower nicotine doses as expressed by a reduction in the number of conditioning trials needed to produce CPP.

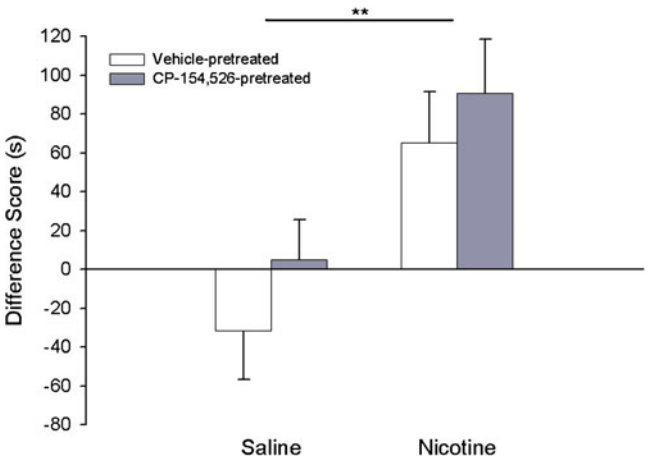

Fig. 3 CPP following conditioning with 0.6 mg/kg nicotine in non-stressed rats pretreated with CP-154,526. *Asterisks* indicate significant difference from saline-conditioned group ($p<0.001$). $n=12$–14 per group

Such results are in line with studies showing that a single stressor exposure 24 h before conditioning enhances amphetamine (Capriles and Cancela 1999), morphine (Dai et al. 2006; Grakalic et al. 2006; Will et al. 1998), and oxycodone (Der-Avakian et al. 2007) CPP. Findings from preclinical studies indicate that acute stress plays an important role in relapse to nicotine seeking as measured by reinstatement of nicotine CPP (Leão et al. 2009) and self-administration (Bruijnzeel et al. 2009; Buczek et al. 1999; Zislis et al. 2007). However, this is the first study that demonstrates a facilitative role of stress in the initial rewarding effects of nicotine. That stress can enhance nicotine's rewarding properties during adolescence, a period during which the majority of smokers initiate use, is especially notable.

Data from Experiments 2 and 3 suggest that CRF-R1 signaling plays a role in stress-induced enhancement of subsequent nicotine reward. Pretreatment with CP-154,526 prior to footshock blocked footshock-induced facilitation of CPP to a moderate nicotine dose but had no effect on CPP to the highest nicotine dose in non-stressed animals. This suggests that under the conditions used here, CRF-R1 signaling specifically during a stressful experience was critical for stress-induced potentiation of nicotine reward. These findings are in line with a study demonstrating that pretreatment with the CRF-R1 antagonist antalarmin blocks stress-induced potentiation of cocaine CPP (Kreibich et al. 2009). A role for CRF-R1 signaling has previously been demonstrated for stress-induced relapse to nicotine seeking, as pretreatment with a CRF-R1 antagonist was shown to prevent stress-induced reinstatement of nicotine self-administration (Bruijnzeel et al. 2009). Our findings extend the role of CRF-R1 to the initiation phase of nicotine addiction and suggest a role for this receptor subtype in stress-induced enhancement of nicotine's rewarding effects.

CP-154,526 pretreatment blocked footshock-induced facilitation of nicotine CPP acquisition but had no effect on footshock-induced freezing. This was an unexpected finding in light of evidence that CRF-R1 antagonists block a variety of behavioral stress responses in rodents, including shock-induced freezing (Bakshi et al. 2002; Deak et al. 1999; Hikichi et al. 2000). It is possible that CRF-R1 blockade did not reduce freezing behavior here due to methodological differences between these previous studies and the present study. For example, the previous studies all used adult rats exposed to higher intensity footshocks than those used here. Freezing behavior was also evaluated after the final shock in the previous studies, whereas freezing was quantified during the 10-min footshock session in the present study.

The present work did not include an investigation of brain regions through which stress might act to enhance nicotine reward. Neural alterations produced by acute stress generally dissipate within a few hours following stressor exposure. Thus, more enduring stress-induced changes are likely to underlie the present results. The mesolimbic DA pathway is thought to be the critical neurobiological substrate for nicotine CPP (Laviolette et al. 2008; Sellings et al. 2008; Spina et al. 2006). There is growing evidence that stress induces lasting adaptations that serve to potentiate activity within this pathway in a manner that may underlie the enhancing effects of stress on nicotine reward. For example, acute restraint stress has been shown to increase burst firing in putative midbrain DA neurons for at least 24 h (Anstrom and Woodward 2005). Moreover, it has been demonstrated that acute forced swim stress enhances strength at excitatory synapses and decreases strength at inhibitory synapses onto midbrain DA neurons for at least 24 h (Niehaus et al. 2010; Saal et al. 2003). It is possible that prior exposure to an acute stressor facilitated nicotine CPP acquisition here via induction of lasting synaptic changes in critical nicotine reward substrates.

Given that the present study used systemic injections for CP-154,526 pretreatment, the brain regions involved in its effects also cannot be determined. CRF is released from the PVN as part of the HPA axis stress response (Smith and Vale 2006) and is likely also released from extrahypothalamic areas (Koob and Heinrichs 1999). There is anatomical and functional evidence for connections between CRF and the mesolimbic DA pathway. CRF-like immunoreactivity has been detected in projections from the PVN and "stress-sensitive" extrahypothalamic areas such as the bed nucleus of the stria terminalis (BNST) and central nucleus of the amygdala (CeA) to the VTA (Rodaros et al. 2007). Acute stress causes release of CRF into the VTA, which contains CRF-R1 (Sauvage and Steckler 2001; Van Pett et al. 2000). It has been suggested that stress-induced CRF release increases firing of VTA DA neurons via CRF-R1 (Wanat et al. 2008). If this is the case, it is possible that CP-154,526 pretreatment prevented stress-induced facilitation of nicotine CPP here by attenuating CRF-R1-mediated effects on adaptations within the mesolimbic DA pathway. Activation of CRF within the NAc, which expresses CRF-R1 (Merchenthaler et al. 1982; Van Pett et al. 2000), may also contribute to the present findings. Though it is not known which NAc cell type(s) contain CRF-R1, it seems reasonable to suggest that it is found on medium spiny projection neurons (MSNs), which are the predominant NAc cell type and possess DA receptors (Wise 2002). Microinjection of CRF into the NAc increases the incentive salience of Pavlovian cues previously associated with reward (Peciña et al. 2006). It would be worthwhile to determine whether stress enhances the salience of nicotine reward-related cues in the CPP paradigm via mechanisms involving NAc CRF-R1.

Another possibility is that CRF-R1 indirectly modulates stress-induced enhancement of nicotine reward via a mechanism involving corticosterone (CORT). Der-Avakian et al. (2005, 2007) found that an acute stressor enhanced the

CORT response to a morphine injection 24 h later and also potentiated subsequent morphine CPP. Temporary suppression of CORT synthesis blocked potentiation of CPP induced by stress 24 h prior and was also shown to block morphine-induced increases in NAc DA (Der-Avakian et al. 2006). Based on these findings, the authors concluded that prior stressor exposure enhances morphine reward by increasing the CORT response to subsequent morphine. Both acute footshock and nicotine produce elevations in plasma CORT (Balfour et al. 1975; Kant et al. 1983). It is possible that as with morphine, intermittent footshock potentiated the nicotine-induced CORT response to subsequent nicotine here and that CP-154,526 pretreatment indirectly attenuated footshock-induced enhancement of nicotine reward via effects on the CORT response to footshock. CORT has positive reinforcing effects and is self-administered by rats (Deroche et al. 1993; Piazza et al. 1993). Release of CORT following stressor exposure may thus also contribute to stress-induced enhancement of drug reward. It would be interesting to determine the effects of an acute stressor on the CORT response to subsequent nicotine as well as the effects of CRF-R1 blockade prior to stressor administration.

It should be noted that the present results could also be explained by non-reward mechanisms, such as the effects of stress on learning. CPP is a learning task based on classical conditioning. Though the relationship between stress and learning in rodents is complex (Sandi and Pinelo-Nava 2007; Shors 2004), acute stressor exposure has been shown to enhance learning in Pavlovian tasks, particularly when there is a delay between the stressor and onset of training. Acute restraint enhances learning of subsequent tone- and context-dependent fear conditioning in mice and rats (Blank et al. 2002; Cordero et al. 2003; Rodríguez Manzanares et al. 2005), and intermittent tailshocks enhance later acquisition of eyeblink conditioning in male rats (reviewed in Shors 2004). One study has shown that stress-induced enhancement of fear learning is prevented by blocking hippocampal CRF-R1 (Radulovic et al. 1999). It is possible that footshock facilitated acquisition of CPP in the present study due to a general enhancement of learning by stress, and that this effect was blocked by CRF-R1 antagonism.

The effects of stress on anxiety must also be noted. Exposure to a single footshock session has been shown to produce increased anxiety-like behavior in rats when tested in a novel environment days to weeks later (reviewed in Stam et al. 2000; Van Dijken et al. 1992a, b). Though both anxiolytic and anxiogenic effects of acute nicotine administration have been documented for adult rats (e.g., File et al. 1998; Irvine et al. 1999), a study by Cheeta et al. (2001) demonstrated anxiolytic effects in adolescents. Though we have previously demonstrated that biased nicotine CPP is not produced due to unconditioned anxiolytic effects of nicotine (Brielmaier et al. 2008), we cannot rule out the possibility of conditioned anxiolysis. Thus, it is possible that prior intermittent footshock enhanced nicotine CPP acquisition due to nicotine's ability to counteract stress-induced anxiety or even by facilitating nicotine's conditioned anxiolytic effects, which along with reward may underlie nicotine's addictive properties. Interestingly, CRF-R1 antagonists have been shown to reduce stress-induced increases in anxiety-like behavior in rats (reviewed in Smagin et al. 2001). Thus, CP-154,526 may have attenuated stress-induced facilitation of CPP acquisition by reducing nicotine's effects on stress-induced anxiety rather than by reducing the effects of stress on the drug's reward efficacy. It should also be noted here that saline injection may have produced residual stress-induced anxiety in saline-conditioned groups as indicated by mild aversion to the white side of the CPP apparatus (i.e., negative difference scores) following conditioning (Figs. 1, 2, and 3). CP-154,526 appears to reduce this mild aversion as evidenced by slightly positive difference scores in non-stressed, antagonist-pretreated groups in Experiments 2 and 3 (Figs. 2 and 3). Such a result further suggests that the present findings might be explained by effects of nicotine and stress on anxiety, an important contributor to nicotine dependence in adolescents (McKenzie et al. 2010).

The present research provides the first evidence that adolescents are susceptible to stress-induced enhancement of nicotine CPP, and that CRF-R1 signaling is involved in this effect. Adolescence is a period of unique vulnerability to nicotine and stress. Initial sensitivity to nicotine's rewarding effects is associated with initiation of regular smoking in this age group, as are stressful life experiences. The identification of stress as a modulator of initial sensitivity to nicotine reward, as well as a role for CRF-R1 in this effect, could inform strategies for prevention and treatment of adolescent nicotine addiction.

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