Nicotine Exposure during Adolescence Induces a Depression-Like State in Adulthood

Sergio D Iñiguez[1,2], Brandon L Warren[1,2], Eric M Parise[1], Lyonna F Alcantara[1], Brittney Schuh[1], Melissa L Maffeo[1], Zarko Manojlovic[1] and Carlos A Bolaños-Guzmán*,[1]

[1]Program in Neuroscience, Department of Psychology, Florida State University, Tallahassee, FL, USA

There is a strong link between tobacco consumption and mood disorders. It has been suggested that afflicted individuals smoke to manage mood, however, there is evidence indicating that tobacco consumption can induce negative mood. This study was designed to investigate whether nicotine exposure during adolescence influences emotionality/behavioral functioning later in life. Adolescent (postnatal days, PD 30–44) male rats were treated with twice-daily injections of nicotine (0, 0.16, 0.32, or 0.64 mg/kg) for 15 consecutive days, and their behavioral reactivity to various behavioral paradigms (the elevated plus maze (EPM), sucrose preference, locomotor activity in the open field, and forced swim test (FST) was assessed 24 h (short term) or 1-month (long term) after exposure. Separate groups of adult rats received nicotine (0.32 mg/kg) to control for age-dependent effects. We report that nicotine exposure during adolescence—but not adulthood—leads to a depression-like state manifested in decreased sensitivity to natural reward (sucrose), and enhanced sensitivity to stress- (FST) and anxiety-eliciting situations (EPM) later in life. Our data show that behavioral dysregulation can emerge 1 week after drug cessation, and that a single day of nicotine exposure during adolescence can be sufficient to precipitate a depression-like state in adulthood. We further demonstrate that these deficits can be normalized by subsequent nicotine (0.32 mg/kg) or antidepressant (ie fluoxetine or bupropion; 10 mg/kg) treatment in adulthood. These data suggest that adolescent exposure to nicotine results in a negative emotional state rendering the organism significantly more vulnerable to the adverse effects of stress. Within this context, our findings, together with others indicating that nicotine exposure during adolescence enhances risk for addiction later in life, could serve as a potential model of comorbidity.

INTRODUCTION

Nicotine dependence through tobacco smoke is the leading preventable cause of death worldwide (Ezzati and Lopez, 2003; Thompson and Fagerstrom, 2006). Although cigarette smoking has declined in recent years in the United States, this has been offset by increases in the consumption of other tobacco products (Connolly and Alpert, 2008; Delnevo et al, 2005), and 30% of those aged 12 and over report past month tobacco use (Greydanus and Patel, 2005; Johnston et al, 2006). The power of nicotine addiction is exemplified by the difficulty in quitting as most addicts wish to quit, but less than 5% succeed (Benowitz, 1999; Hatsukami et al, 2008), and despite devastating health consequences, close to 50% of those hospitalized for tobacco-related illnesses

relapse to smoking soon after leaving the hospital (Dornelas and Thompson, 2007; Thorndike et al, 2008).

The neurobiological actions of nicotine are similar to the psychomotor stimulants cocaine and amphetamine (Laviolette and van der Kooy, 2004): it interacts with neurons in the ventral tegmental area (VTA) and nucleus accumbens (NAc)—brain regions crucial for reward and addiction (Corrigall et al, 1992), enhances locomotor activity (Zubaran et al, 2000), and regulates the reinforcing properties of abused drugs (Zachariou et al, 2001). Because repeated exposure to stimulants can predispose individuals toward drug addiction (Chao and Nestler, 2004), most research has focused on delineating the mechanisms underlying nicotine dependence, treatment, and prevention (Benowitz, 2008; Picciotto, 2003).

The prevalence of nicotine dependence is significantly higher among individuals with mood disorders (Grant et al, 2004; Leonard et al, 2001), and because nicotine can alleviate negative affect, afflicted individuals may initiate smoking to regulate mood through a self-medication process (Breslau et al, 2004b; Dani and Harris, 2005; Markou et al, 1998). Conversely, there is evidence suggesting

*Correspondence: Dr CA Bolaños-Guzmán, Program in Neuroscience, Department of Psychology, Florida State University, 1107 West Call Street, Tallahassee, FL 32306-4301, USA, Tel: + 1 850 644 2627, Fax: + 1 850 645-7518, E-mail: bolanos@psy.fsu.edu
[2]These authors contributed equally to this work.

that cigarette smoking and level of nicotine dependence might precipitate mood-related disorders, as studies show that smokers have a greater risk of becoming depressed than nonsmokers (Breslau *et al*, 1998; John *et al*, 2004; Klungsoyr *et al*, 2006). Most adult smokers (80%) started during adolescence (Lenney and Enderby, 2008; Warren *et al*, 2008), and adolescents who smoke daily have a 70% prevalence of psychiatric disorders (Choi *et al*, 1997; Kandel *et al*, 1997), and elevated rates of comorbidity in adulthood (Clark *et al*, 1998; Patton *et al*, 2006; Volkow, 2004).

Given the co-occurrence of nicotine use/dependence and mood disorders, that smoking is mostly initiated during adolescence, and that nicotine can induce stress and precipitate depression and anxiety (O'Loughlin *et al*, 2002; Parrott, 1999; Volkow, 2004), it is crucial to delineate whether nicotine exposure during adolescence results in depression-like behaviors later in life. We treated adolescent male rats with twice-daily injections of saline (VEH) or nicotine (0.16, 0.32, and 0.64 mg/kg) for 15 days (postnatal days (PD) 30–44). After VEH or nicotine treatment, behavioral reactivity to the elevated plus maze (EPM), forced swim test (FST), sucrose preference, and the open field was assessed 24 h or 1 week after the last nicotine injection, or when rats reached adulthood (PD 70 +). This approach was taken because nicotine interacts with mesolimbic reward pathways that are part of the neural circuitry essential in the control of mood under normal conditions (Nestler and Carlezon, 2006).

MATERIALS AND METHODS

Animals

Preadolescent (PD 25 at day of arrival) and adult (250–275 g) male Sprague–Dawley rats were obtained from Charles River Laboratories (Raleigh, NC). Rats were habituated to the animal facility for 5 days. The age at the start and duration of nicotine exposure in adolescent rats (PD 30–44) was selected because it roughly approximates adolescence (ie age 12–18 years) in humans (Andersen and Navalta, 2004; Spear, 2000; Spear and Brake, 1983). Rats were housed two per cage in clear polypropylene boxes containing wood shavings without external objects in an animal colony maintained at 23–25°C on a 12 h light/dark cycle in which lights were on between 0700 and 1900 hours. Rats were provided with food and water *ad libitum*.

Drugs

(−)-Nicotine hydrogen tartrate (NIC), fluoxetine hydro-chloride (FLX), and bupropion hydrochloride (BPN) were obtained from Sigma (St Louis, MO). NIC was dissolved in sterile physiological saline (0.9% sodium chloride; VEH), and the solution was adjusted to a pH of 7.4. FLX and BPN were dissolved in sterile distilled water. NIC and BPN were administered in a volume of 1 ml/kg, whereas FLX was administered in a volume of 2 ml/kg. NIC doses are expressed as the free base, whereas the doses for FLX and BPN are reported as the salt form of the drug.

Drug Treatments and Experimental Design

An initial experiment was conducted to determine the short- and long-term behavioral effects of various doses of NIC using the open-field test (OFT) and the FST. Adolescent rats received subcutaneous (s.c.) injections of VEH or NIC (0.16, 0.32, and 0.64 mg/kg) twice daily (4 h apart) for 15 consecutive days (last day: PD 44). At 24 h after treatment (short-term group), locomotor activity (ie distance traveled) in the open field, or behavioral reactivity to forced swimming stress, was assessed. Rats exposed to the open field were left undisturbed for 1 month (long-term group) after the test, and then exposed to the FST in adulthood (PD 70 +). Although reliable effects in the FST were observed with both of the higher doses, we chose the 0.32 mg/kg NIC dose to further assess its influence on rats' behavioral reactivity to other emotion-eliciting stimuli, and to determine whether this dose would have similar effects in NIC-naive adult rats. We also chose this dose because it is within the range of those used in models assessing neurobiological responses to NIC *in vivo* (Collins *et al*, 2004; Matta *et al*, 2007; Vastola *et al*, 2002). Accordingly, separate groups of adolescent and adult rats (300–320 g at the start of drug treatment) were exposed to NIC (0.32 mg/kg) for 15 days and their behavioral reactivity was assessed 24 h or 1 month after treatment. More specifically, rats assigned to short-term group were tested in a single noninvasive behavioral paradigm (ie locomotor activity, EPM, or sucrose preference), and then left undisturbed for 1 month at which time they were tested on behavioral paradigms that they had not been exposed to earlier. Rats tested in the FST 24 h after NIC were not exposed to further testing. Regardless of the experimental condition, rats were not exposed to more than two behavioral paradigms, and were never tested again after FST.

For the reversal experiments using the FST, rats were treated with VEH or NIC (0.32 mg/kg) as described above and assigned to post-treatment groups receiving SAL, NIC, FLX, or BPN. Rats assigned to the NIC post-treatment group received a single NIC (0.32 mg/kg) injection 10 min before exposure to day 2 of the FST. Rats assigned to the FLX or BPN groups received a daily injection of FLX (10 mg/kg) or BPN (10 mg/kg) for 5 days (PD 70–75) before being exposed to the FST. That is, rats received their last injection of FLX or BPN 24 h before day 1, or 48 h before day 2 of the FST. All behaviors, except for sucrose preference and locomotor activity, were recorded with a video camera located on the ceiling of separate testing rooms. In the long-term group, there was a period of at least 24–36 h between each behavioral test. Behavioral observations and analyses were carried out by observers with no knowledge of the treatment conditions of each rat. Experiments were conducted in compliance with the *Guidelines for the Care and Use of Laboratory Animals* (National Institute of Health, 1996), and approved by Florida State University Animal Care and Use Committee.

Locomotor Activity

VEH- and NIC-treated adolescent and adult rats were tested to examine the short- and long-term effects of NIC on

locomotor activity as indexed by distance traveled (cm) in an open-field apparatus ($63 \times 63 \times 26$ cm) for 1 h.

Elevated Plus Maze

VEH- and NIC-treated adolescent and adult rats were tested for 5 min on the EPM, a behavioral model of anxiety-like behavior. The maze was made of gray plastic and consisted of two perpendicular, intersecting runways (12 cm wide $\times$ 100 cm long). One runway had tall walls (40 cm high) or 'closed arms,' whereas the other one had no walls or 'open arms.' The arms were connected together by a central area, and the maze was elevated 0.5 m from the floor. Testing was conducted between 0900 and 1300 hours under controlled light conditions (~ 90 lux). At the beginning of the test, rats were placed in the central area, facing one of the open arms, and the cumulative time spent in the open arms was videotaped. We also assessed self-grooming in the 'closed arms' because rats engage in repetitive grooming in response to anxiogenic stimuli (Spruijt et al, 1988).

Sucrose Preference

This test consisted of a two-bottle choice paradigm in which rats are given the choice between consuming water vs sucrose. This paradigm has been used extensively to assess the effects of stress-induced anhedonia (Papp et al, 1991; Sampson et al, 1992). Rats were habituated to drink water from two bottles for 5 days. Each day the water bottles were switched (left or right). At the start of the experiment (day 5) potential bottle and side preference(s) was assessed by measuring water/water intake and balancing the position of the bottles (left or right). On day 6, rats were exposed to sucrose (0, 1%) for 24 h. The position of the sucrose bottle (left or right) was again balanced between the groups/cages. More specifically, the position of the water and sucrose bottles (left or right) was switched every 30 min from 1900 to 2200 hours. After the 3 h period, rats were left undisturbed and their overnight fluid consumption was measured the next morning (0800 hour). The preference for sucrose over water (ie sucrose intake divided by the sum of total water plus sucrose intake (sucrose/(water + sucrose)) was used as a measure for rats' sensitivity to reward. No differences in sucrose preference or liquid intake were found during the 3 h period, thus the water and sucrose consumption data analyzed were those taken at 1900 and 0800 hours.

Forced Swim Test

The FST is a 2-day procedure in which rats are forced to swim under conditions they cannot escape. On day 1, rats are forced to swim. Initially, they engage in escape-like behaviors but eventually adopt a posture of immobility in which they make only the movements necessary to maintain their head above water. When retested 24 h later, rats become immobile very quickly; however, antidepressant treatment between the forced swim exposures can significantly increase their escape-like behaviors, an effect that has been correlated with antidepressant activity in humans (Cryan et al, 2002). At the start of the experiment, rats were placed in plastic cylinders (75×30 cm) filled to 54 cm depth

with 25°C water and forced to swim for 15 min. At the end of this period, rats were removed from the water, dried with towels, and placed in a warmed enclosure for 30 min. All cylinders were emptied and cleaned between rats. Rats were retested for 5 min under identical conditions 24 h after the forced swim (day 2). In this study, the latency to become immobile, total immobility, swimming, immobility, and climbing counts (Carlezon et al, 2003; Detke et al, 1995) were the dependent variables. Behavioral counts were taken at 5-s intervals during the 5-min retest. Latency to immobility was defined as the time at which the rat first initiated a stationary posture that did not reflect attempts to escape from the water (Lucki, 1997). To qualify as immobility, this posture had to be clearly visible and maintained for $\geqslant 2.0$ s.

Statistical Analyses

Assignment of subjects to the various testing conditions was random. Behavioral data were analyzed using mixed-design (between and within variables) repeated analyses of variance (ANOVAs) followed by Fisher's least-significant difference post hoc test. When appropriate, additional one-way ANOVAs or Student's t-tests were used to determine statistical significance of preplanned comparisons. Data are expressed as the mean ± SEM. In all cases, statistical significance was defined as $p < 0.05$.

RESULTS

Chronic NIC Exposure on Weight Gain in Adolescent and Adult Rats

Figure 1a shows the effects of chronic VEH or NIC (0.16, 0.32, 0.64 mg/kg) treatment on weight gain in adolescent rats. Repeated-measures ANOVA (for day of injection) revealed that NIC treatment significantly influenced body weight across days (within-subject main effect: $F_{(14, 420)} = 258.03$, $p < 0.0001$) and drug-treatment (between-subject main effect: $F_{(3, 420)} = 50.47$, $p < 0.001$). Although body weight increased with age across all groups, NIC-treated rats showed significantly lower weights when compared to controls ($p < 0.05$) as of the fourth day of treatment ($n = 8$ per group). Overall, body weight between VEH- and NIC-treated groups did not differ at the time of behavioral testing in adulthood. Data presented in the inset of Figure 1a are representative of rats treated with 0.32 mg/kg NIC during adolescence, and tested for sucrose preference in adulthood. As can be seen in Figure 1b, NIC (0.32 mg/kg) or VEH treatment did not affect weight gain in adult rats ($n = 7$ per condition).

Short- and Long-term Effects of NIC Exposure During Adolescence on Locomotor Activity

VEH- and NIC-treated adolescent rats were placed in an open-field apparatus 24 h after the last injection. Figure 2a shows that the effects of NIC exposure varied across time (within-subject main effect: $F_{(11, 396)} = 141.28$, $p < 0.0001$), and as a function of NIC dose (treatment main effect: $F_{(3, 396)} = 14.81$, $p < 0.0001$). In general, rats treated with the 0.32 mg/kg NIC dose had longer distance traveled for the
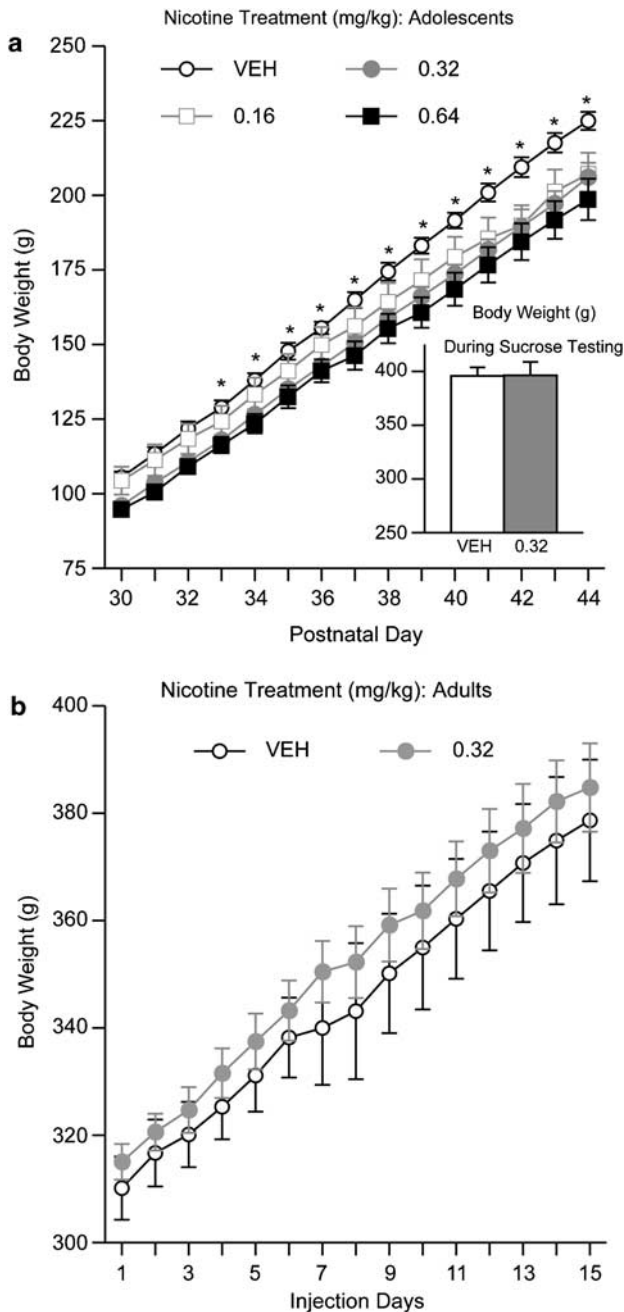
Figure 1 Effects of (−)-nicotine hydrogen tartrate (NIC) on weight gain (a and b). (a) Adolescents ($n = 32$): body weight increased across days regardless of condition, and NIC treatment resulted in significantly lower weight gain when compared to control rats. *Significantly different from NIC-treated rats ($p < 0.05$). (b) Adults ($n = 14$): no difference in body weight after NIC (0.32 mg/kg) was apparent. Data are presented average weight gain across days and drug treatment (mean ± SEM, in gram).

first 30 min of the test. As can be seen in the inset of Figure 2a, rats exposed to 0.32 NIC traveled greater distance overall (treatment main effect: $F_{(3, 33)} = 2.88$, $p < 0.05$), especially when compared to the VEH ($p < 0.05$) and the 0.16 NIC ($p < 0.05$) treatment groups ($n = 9–10$ per condition).

Separate groups of VEH- and NIC-treated adolescent rats were tested in the open field when they reached adulthood

(PD 70 + ; Figure 2b). The effects of NIC on distance traveled varied across time (within-subject main effect: $F_{(11, 264)} = 99.45$, $p < 0.0001$), and as a function of NIC dose (treatment main effect: $F_{(11, 264)} = 7.5$, $p < 0.0001$). However, a treatment effect was only apparent at 20 min in the 0.64 mg/kg NIC group. The inset of Figure 2b shows no differences in overall distance traveled across any of the treatment groups ($n = 6–8$ per condition).

Short- and Long-term Effects of NIC Exposure on Locomotor Activity in Adult Rats

VEH- and NIC-treated adult rats were placed in an open-field apparatus 24 h or 1 month after the last injection (Figure 2c and d). Chronic exposure to 0.32 mg/kg NIC did not influence distance traveled in the open field 24 h (Figure 2c; $n = 14$), or 1 month after NIC treatment (Figure 2d; $n = 12$).

Short- and Long-term Effects of NIC Exposure During Adolescence on Forced Swimming Behavior

We used the FST to study rats' responses to stressful conditions either 24 h or when they reached adulthood (1 month) after the last VEH or NIC injection (Figure 3a–f). There were no significant differences in latency to immobility, or total immobility, in adolescent rats tested 24 h after NIC treatment on day 2 of the FST (Figure 3a and b). Nevertheless, NIC exposure influenced immobility ($F_{(3, 29)} = 8.73$; $p < 0.0001$), swimming ($F_{(3, 29)} = 3.29$; $p < 0.036$), and climbing ($F_{(3, 29)} = 11.09$; $p < 0.0001$) counts (Figure 3c). For instance, adolescent rats treated with 0.32 or 0.64 NIC had significantly lower levels of immobility ($p < 0.05$), and higher levels of swimming ($p < 0.05$) and climbing ($p < 0.05$) counts than VEH- or the 0.16 mg/kg NIC-treated rats ($n = 7–9$ per condition).

When assessing the long-term effects of adolescent exposure to NIC, a different behavioral profile emerged indicative of a depression-like effect (Figure 3d–f). Exposing adolescent rats to NIC resulted in significantly lower latencies to immobility in adulthood ($F_{(3, 35)} = 4.23$; $p < 0.013$; Figure 3d). More specifically, rats treated with 0.32 or 0.64 mg/kg NIC had significantly lower latencies to immobility ($p < 0.05$) than VEH- or the 0.16 mg/kg NIC-treated rats (Figure 3d). In addition, NIC-treated rats (0.32 and 0.64 mg/kg) had significantly higher levels of total immobility ($F_{(3, 35)} = 11.7$; $p < 0.001$) when compared to VEH and the 0.16 mg/kg dose (Figure 3e); significantly higher immobility ($F_{(3, 35)} = 9.36$; $p < 0.0001$; Figure 3f), and lower swimming counts ($F_{(3, 35)} = 21.15$; $p < 0.0001$), than VEH- or the 0.16 mg/kg NIC-treated rats (Figure 3f; $n = 9$ per condition).

Short- and Long-term Effects of NIC Exposure in Adolescent and Adult Rats on Sucrose Preference

As can be seen in Figure 4, there were no differences in side preference or water intake across the various experimental groups. A one-way ANOVA did not yield significant differences in sucrose preference in adolescent rats when tested 24 h after 0.32 mg/kg NIC (Figure 4a; VEH-treated $n = 8$; NIC-treated $n = 8$). However, *t*-test revealed that
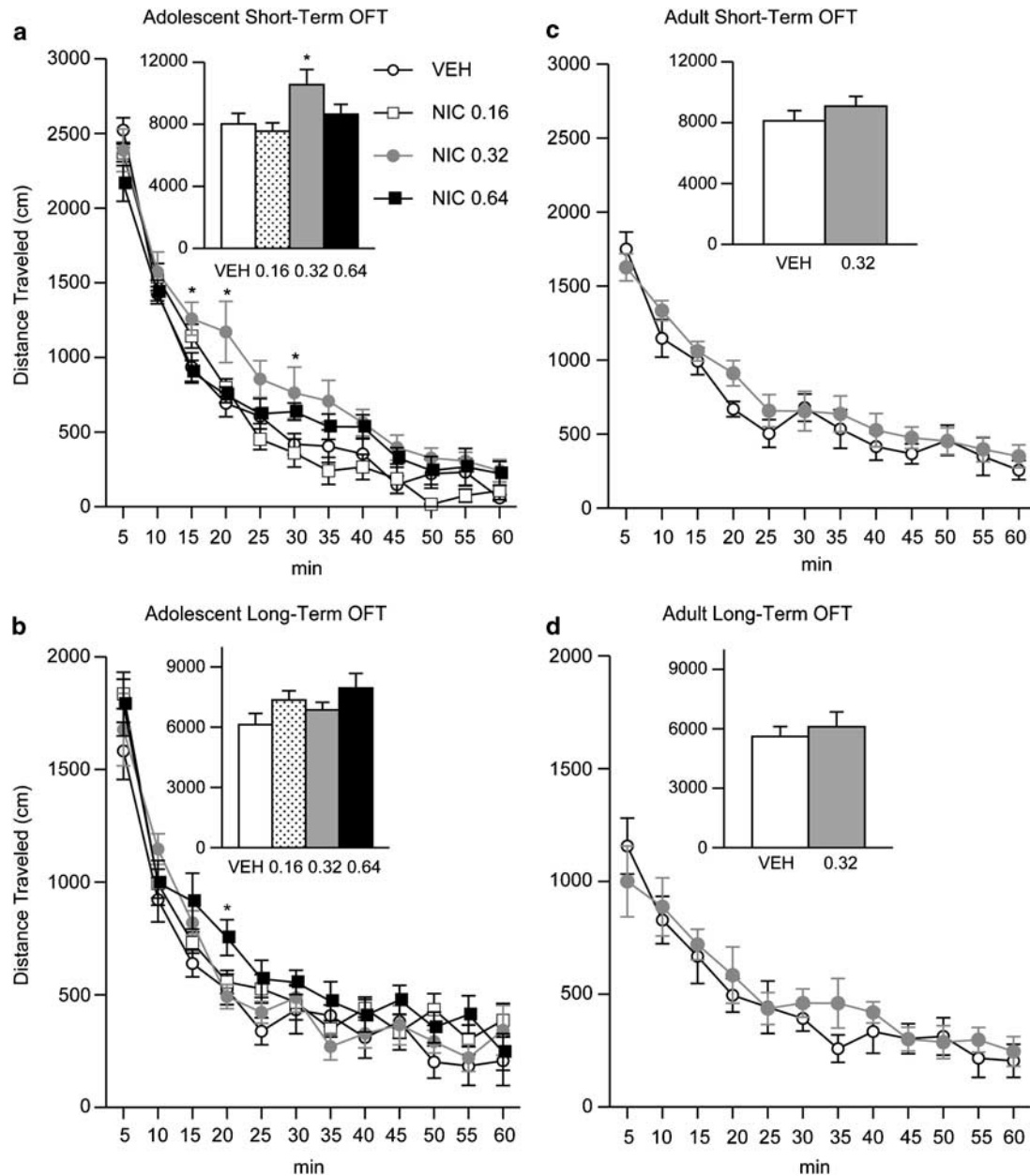
Figure 2 Effects of (−)-nicotine hydrogen tartrate (NIC) exposure on locomotor activity (a–d). (a) Short-term adolescent (n = 37): rats exposed to 0.32 mg/kg NIC and tested 24 h after treatment had significantly higher levels of distance traveled for the first 30 min of the test (*p < 0.05: different from VEH and the 0.16, 0.64 NIC groups). (b) Long-term adolescent (n = 26): no difference in locomotor activity was observed in adult rats exposed to VEH or NIC during adolescence. (c) Short-term adults (n = 14): NIC (0.32 mg/kg) had no effects on distance traveled in adult rats tested 24 h after exposure. (d) Long-term adults (n = 12): NIC (0.32 mg/kg) had no effects on distance traveled in adult rats tested 4 weeks after exposure. Data are presented as mean total distance traveled (mean ± SEM, in cm).

these rats showed a significant increase in total liquid intake ($t_{(14)} = 5.7$; $p < 0.001$; Figure 4b, left panel) and sucrose consumption ($t_{(14)} = 4.88$; $p < 0.004$; Figure 4c, left panel) as compared to controls. A separate one-way ANOVA revealed a somewhat different pattern of results in rats treated during adolescence and tested in adulthood ($F_{(1, 18)} = 12.8$; $p < 0.003$; Figure 4a–c, right panels). These rats exhibited a modest, yet significant decrease in sucrose preference ($p < 0.05$), and had no statistical significant changes in total liquid intake (Figure 4b, right panel) or total sucrose consumed (Figure 4c, right panel) when compared to VEH rats (VEH-treated n = 10; NIC-treated n = 10). As can be

seen in Figure 4d–f, there were no differences in either sucrose preference or any other parameter tested between the groups exposed to VEH or NIC in adulthood (short-term group: VEH-treated n = 7; NIC-treated n = 7; long-term group: VEH-treated n = 7; NIC-treated n = 7).

Short- and Long-Term Effects of NIC Exposure in Adolescent and Adult Rats on Anxiety-Like Behaviors in the Elevated Plus Maze

We also studied the short- and long-term effects of NIC (0.34 mg/kg) exposure during adolescence on anxiety-like
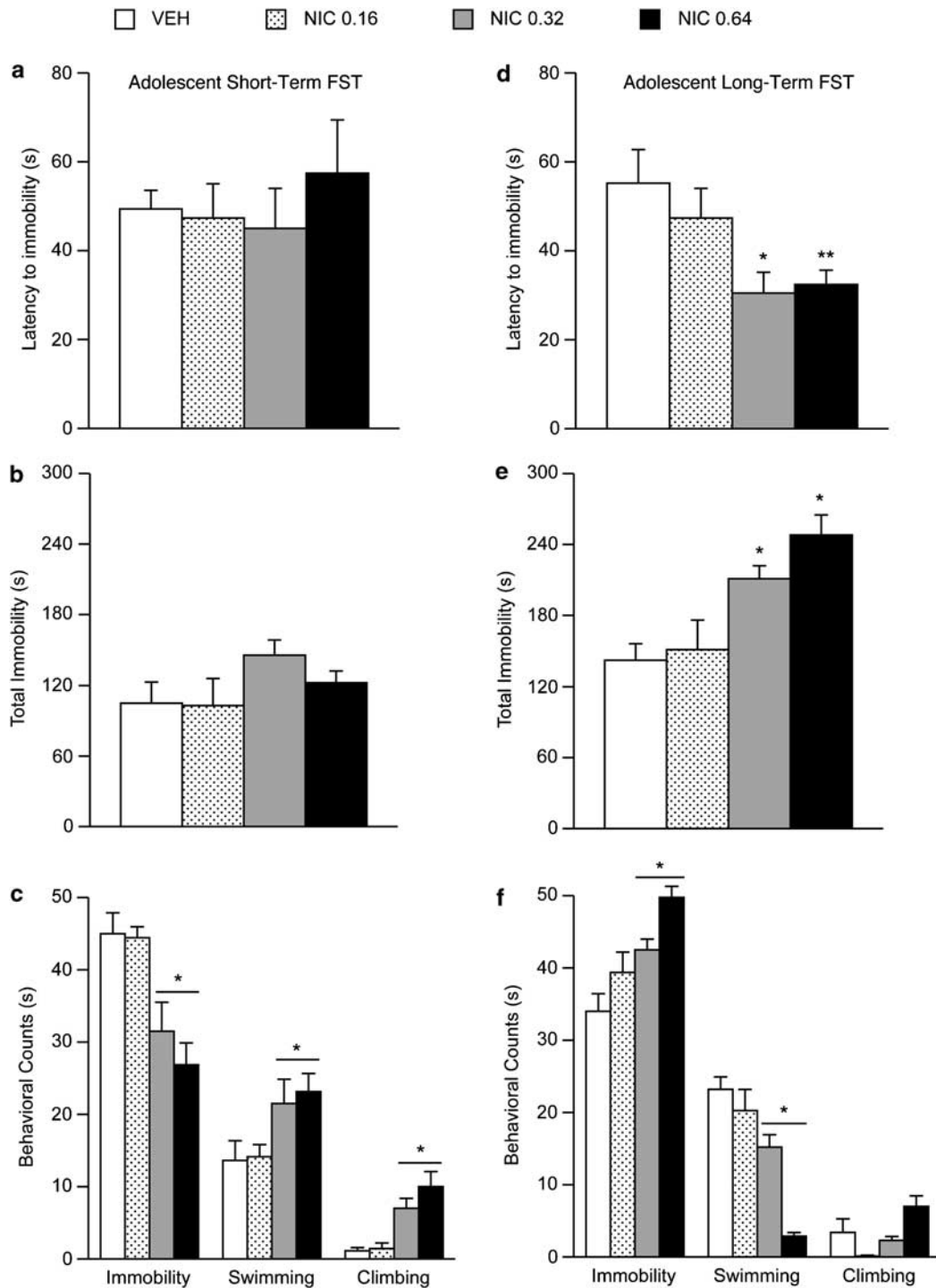
□ VEH   ▨ NIC 0.16   ▧ NIC 0.32   ■ NIC 0.64

Figure 3 Effects of (−)-nicotine hydrogen tartrate (NIC) exposure during adolescence on forced swimming behaviors (a–f). (a) Short-term ($n = 30$): no significant differences in latency or (b) total immobility were found in NIC-exposed adolescent rats tested 24 h after exposure. (c) Rats exposed to 0.32 or 0.64 mg/kg NIC had significantly lower immobility, and significantly higher swimming and climbing counts (*$p < 0.05$: different from VEH and 0.16 NIC). (d) Long-term ($n = 36$): adult rats exposed to 0.32 (*$p < 0.05$) or 0.64 (**$p < 0.05$) mg/kg NIC during adolescence had significantly shorter latency to immobility than the VEH and the 0.16 NIC rats on day 2 of the forced swim test (FST). (e) These rats show higher levels of total immobility and (f) immobility counts, and lower levels of swimming and climbing (*$p < 0.05$: different from VEH and 0.16 NIC). Data are presented as latencies to become immobile, total immobility (in seconds), swimming, immobility, and climbing counts (mean ± SEM).

behavior using the EPM (Figure 5a–c; VEH = 7; NIC = 8). Time spent in the open arms of the EPM did not differ between the VEH and NIC groups tested 24 h after the last injection. In addition, no differences in open arm entries or self-grooming were apparent (Figure 5b and c, left panel).

We then assessed the long-term effects of NIC exposure during adolescence (Figure 5a–c, right panel; VEH = 9; NIC = 8). Time spent in the open arms of the EPM was influenced by NIC treatment, as rats exposed to NIC during adolescence spent significantly less time in the open arms
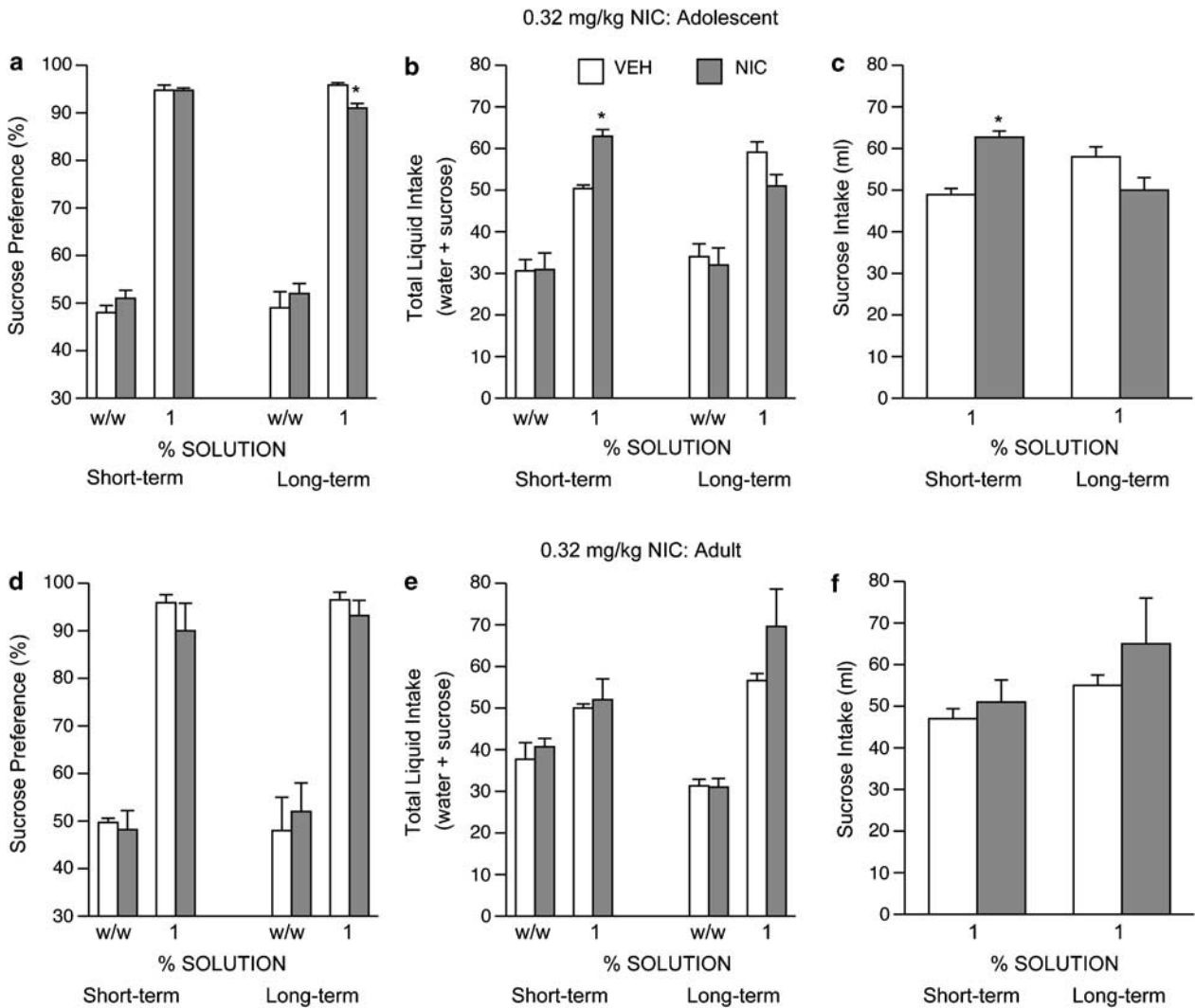
0.32 mg/kg NIC: Adolescent

0.32 mg/kg NIC: Adult

Figure 4 Effects of (–)-nicotine hydrogen tartrate (NIC) exposure in adolescent and adult rats on sucrose preference (a–f). No differences in bottle/side preference (w/w) were observed across the various groups. Short term ($n = 16$): (a, left panel) no differences in sucrose preference detected in adolescent rats 24 h after NIC exposure. (b, left panel) these rats showed significantly higher levels of total liquid (sucrose + water) intake ($*p < 0.05$), and (c, left panel) total sucrose consumed ($*p < 0.05$) than the VEH-treated rats. Long term ($n = 20$): (a, right panel) adolescent treatment with NIC (0.32 mg/kg) resulted in a significant decrease in sucrose preference in adulthood ($*p < 0.05$). These rats showed no differences in total liquid (sucrose + water) intake (b, right panel), or (c, right panel) in total sucrose consumed. Adult rats short term ($n = 14$): no differences in sucrose preference detected (d–f, left panel). Adult rats long-term ($n = 14$): no differences in sucrose preference detected (d–f, right panel). Data are presented as percent preference or total ml consumed between VEH- and NIC-exposed rats.

than their controls ($F_{(1, 15)} = 4.67$; $p < 0.04$; Figure 5a, right panel). In addition, rats treated with NIC during adolescence had a significantly lower percentage entries into the open arms ($F_{(1, 15)} = 62.23$; $p < 0.0001$; Figure 5b, right panel), and significantly higher levels of self-grooming in the closed arms of the EPM than controls ($F_{(1, 15)} = 5.17$; $p < 0.038$; Figure 5c, right panel). NIC exposure during adulthood did not influence anxiety-related behaviors at any of the time points assessed (Figure 5d–f).

Short- and Long-Term Effects of NIC Exposure in Adolescent and Adult Rats on Forced Swimming Behavior

Figure 6a–f shows the short- and long-term effects of chronic 0.32 mg/kg NIC exposure in adolescent and adult rats. It must be noted that the short- and long-term data presented for rats treated during adolescence were taken from Figure 3 for comparison purposes. As can be seen in Figure 6, chronic NIC did not have any short- ($n = 16$) or long-term ($n = 18$) effects in adult rats exposed to the FST.

NIC Exposure During Adolescence: 1 Week After the Last NIC Injection on Forced Swimming Behavior

Because NIC exposure had no effects on the FST 24 h after the last injection in adolescent rats, we assessed whether the long-term effects induced by NIC in the FST would be apparent 1 week after the last injection. Chronic NIC (0.32 mg/kg) exposure during adolescence resulted in a significant decrease in latency to immobility on day 2 of the FST ($F_{(1, 16)} = 11.94$; $p < 0.0032$) 1 week after the last NIC injection (Figure 7a, $n = 18$). In addition, these rats had significantly higher immobility counts ($F_{(1, 16)} = 12$; $p < 0.003$), and significantly
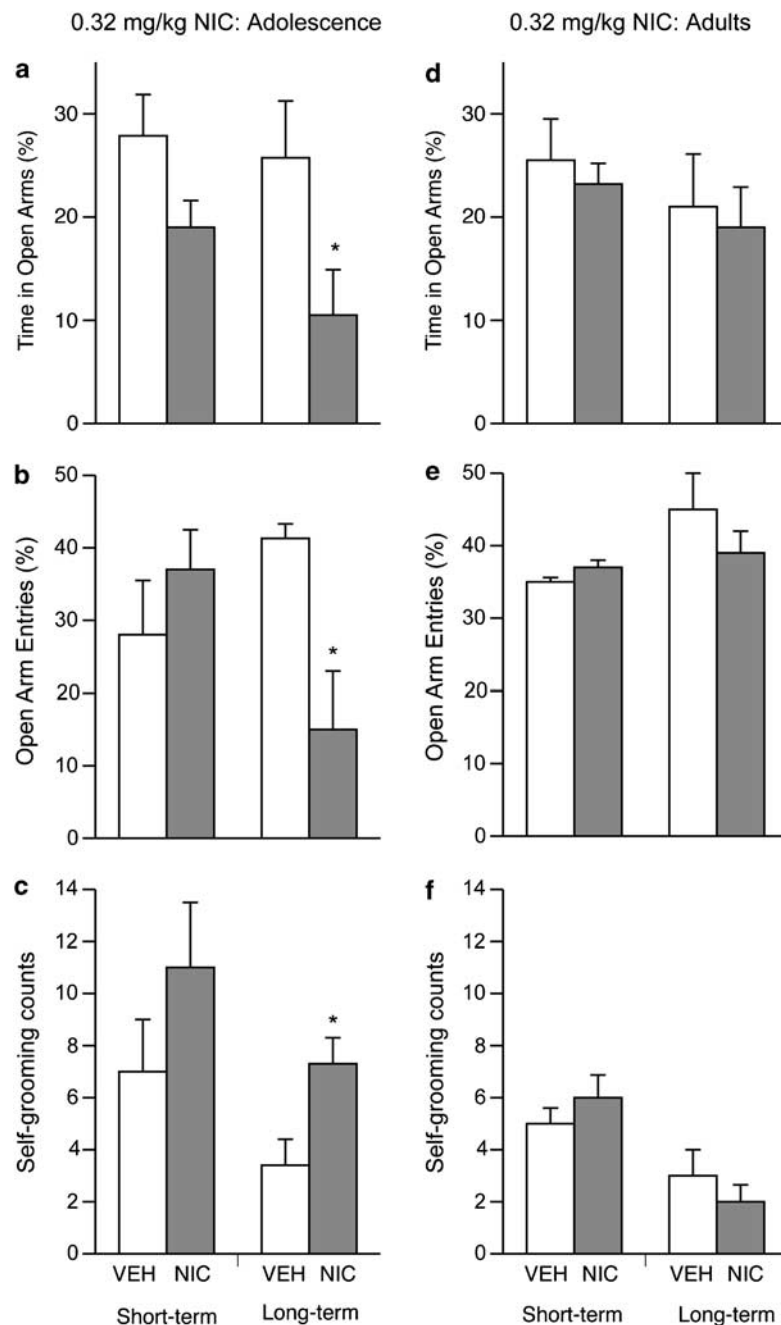
0.32 mg/kg NIC: Adolescence   0.32 mg/kg NIC: Adults

Figure 5 Effects of (−)-nicotine hydrogen tartrate (NIC) exposure in adolescent and adult rats on anxiety-like behaviors in the elevated plus maze (EPM) (a–f). Short term ($n = 15$): (a, left panel) no differences in time spent in the open arms of the EPM were observed in adolescent rats. No changes in entries into the open arms (b, left panel) or self-grooming in the closed arms (c, left panel) were detected. Long term ($n = 16$): (a, right panel) adolescent rats treated with NIC and tested in adulthood showed a significant decrease in time spent in the open arms of the EPM when compared to controls (*$p < 0.05$). (b, right panel) these rats demonstrated significant decreases in the percent of entries into the open arms of the EPM (*$p < 0.05$), and (c, right panel) increased self-grooming behavior (*$p < 0.05$) as compared to controls. Adult rats short term ($n = 12$): NIC exposure had no short-term effects in adult rats (d–f, left panel). Adult rats long term ($n = 14$): NIC had no long-term effects in adult rats (d–f, right panel). Data are presented as percent time spent (mean ± SEM) and percent entries into the open arms, and as self-grooming counts (mean ± SEM) in the closed arms.

lower swimming counts ($F_{(1, 16)} = 11.81$; $p < 0.0034$) than the VEH-treated rats (Figure 7c).

NIC Exposure During Adolescence: Long-Term Effects After a Single Day of NIC Exposure on Forced Swimming Behavior

It has been shown that synaptic changes are apparent in young rats after a brief exposure to NIC (Mansvelder and McGehee, 2000). Thus, we assessed whether a single day of NIC exposure would have long-lasting behavioral effects in adolescent rats (Figure 7d–f). Rats exposed to a single day of NIC (ie two injections, 0.32 mg/kg) during adolescence (PD 30) and tested in adulthood (PD 85) did not exhibit a statistical significant decrease in latency to immobility on day 2 of the FST (Figure 7d; $n = 8$–10 per condition). These NIC-treated rats had significantly higher levels of total

immobility ($F_{(1, 16)} = 5.45$; $p < 0.033$; Figure 7e). In addition, NIC-exposed rats had significantly lower swimming counts ($F_{(1, 16)} = 17.76$; $p < 0.0007$) than the VEH-treated rats (Figure 7f). No changes were apparent in immobility or climbing counts between groups.

Long-Term Effects of NIC Exposure on the Forced Swim Test: Reversal by NIC and Antidepressant Treatment

In this study we assessed whether NIC, FLX, or BPN could reverse the long-term behavioral deficits observed in the FST in adult rats exposed to 0.32 mg/kg NIC during adolescence (Figure 8a–c; $n = 48$, ie VEH/SAL = 8; NIC/SAL = 7; VEH/NIC = 10; NIC/NIC = 6; NIC/FLX = 10; NIC/BPN = 7). Figure 8a shows that behavioral responsivity on day 2 of the FST varied as a function of adolescent and adult drug treatment (pretreatment × posttreatment interaction: $F_{(1, 42)} = 10.635$; $p < 0.002$). More specifically, rats treated with NIC during adolescence and receiving a SAL injection on day 2 of the FST (ie NIC/SAL group) had significantly lower latencies to immobility than the VEH/SAL group ($p < 0.05$), whereas the VEH/NIC rats had

significantly higher latencies to immobility than the VEH/SAL or the NIC/SAL groups ($p < 0.05$), respectively. These results replicate our original observation that NIC exposure during adolescence results in a depression-like effect in the FST, and further confirm the results of others indicating that NIC decreases immobility in the FST.

An acute injection of NIC (0.32 mg/kg, s.c.) 10 min before the test reversed the effects of NIC exposure during adolescence ($p < 0.05$: as compared to the NIC/SAL and VEH/NIC groups). A single injection of FLX (10 mg/kg) or BPN (10 mg/kg) had no effect on latency to immobility (data not shown), whereas chronic (one-daily injection for 5 days) treatment to FLX or BPN reversed the effects induced by NIC exposure during adolescence in the FST ($p < 0.05$: as compared to the NIC/SAL group). The FLX dose was chosen based on previous dose–response data from our laboratory indicating that lower doses (2.5 or 5 mg/kg) do not influence behavioral responding in the FST (Bolaños et al, 2008). The BPN dose was chosen based on previous reports demonstrating that 10 mg/kg BPN is a low dose that does not enhance locomotor activity in rats previously exposed to VEH or NIC (Wilkinson and Bevins, 2007; Wilkinson et al, 2006),
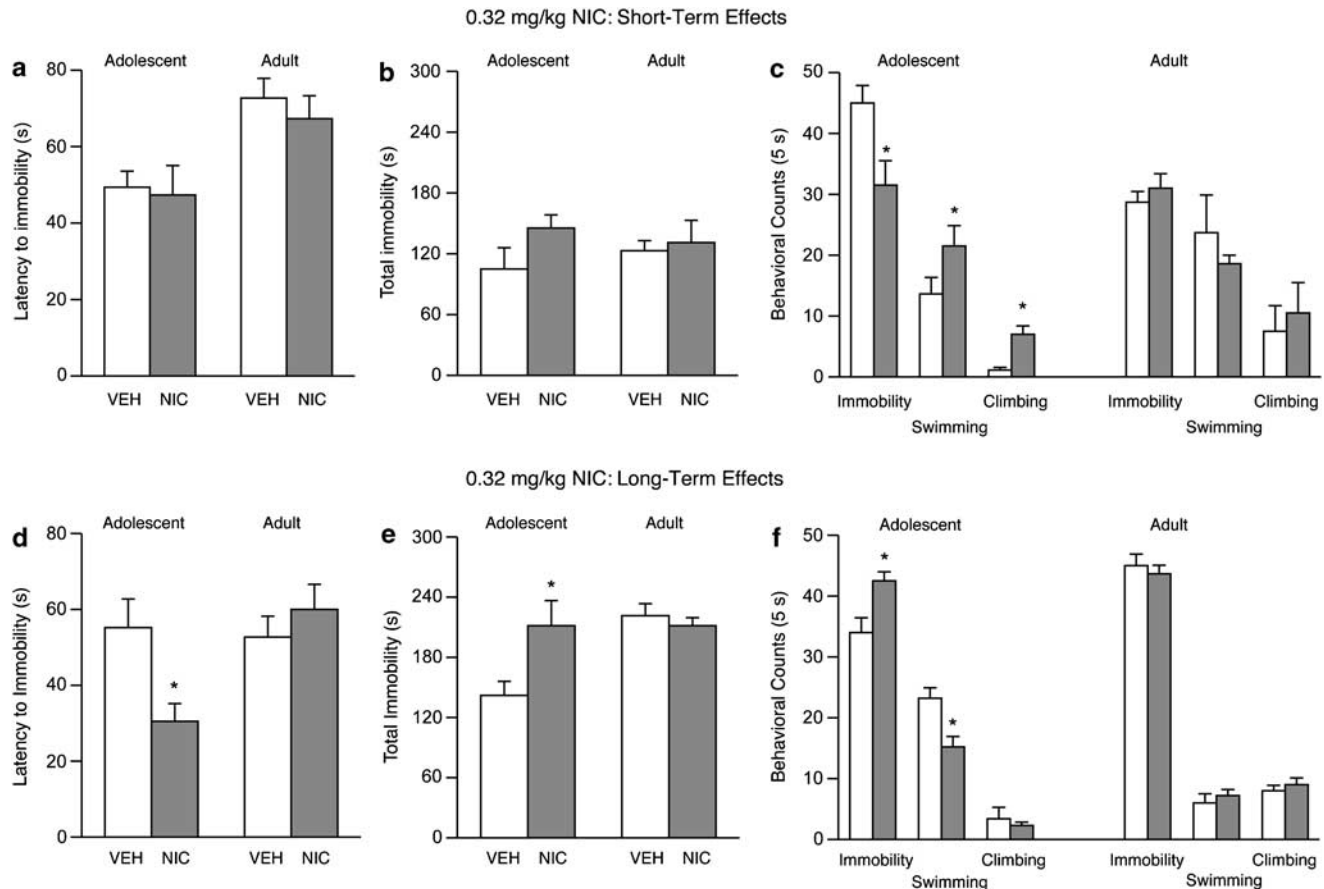

Figure 6 Effects of (−)-nicotine hydrogen tartrate (NIC) exposure in adolescent and adult rats on behavioral responsivity to forced swim stress (a–f). Note that the data for adolescent rats (short and long term) are the same as presented in Figure 3 (0.32 mg/kg). Short term ($n = 16$): (a, left panel) no significant differences in latency to immobility or (b, left panel) total immobility were found in adolescent rats exposed to 0.32 mg/kg NIC and tested 24 h after treatment. (c, left panel) these rats had significantly lower levels of immobility, and higher swimming and climbing counts (*$p < 0.05$: different from VEH). Long term ($n = 20$): (d, left panel) rats exposed to NIC during adolescence and tested as adults had significantly shorter latency to immobility than the VEH rats (*$p < 0.05$) on day 2 of the forced swim test (FST). (e, right panel) these rats also show significantly higher levels of total immobility and (f, right panel) immobility counts, whereas showing lower levels of swimming and climbing (*$p < 0.05$: different from VEH). No significant differences were found in rats treated with NIC as adults and tested at the short- ($n = 16$) or long-term ($n = 18$) time points (a–f). Data are presented as latencies to become immobile, total immobility (in seconds), or swimming counts (mean ± SEM).
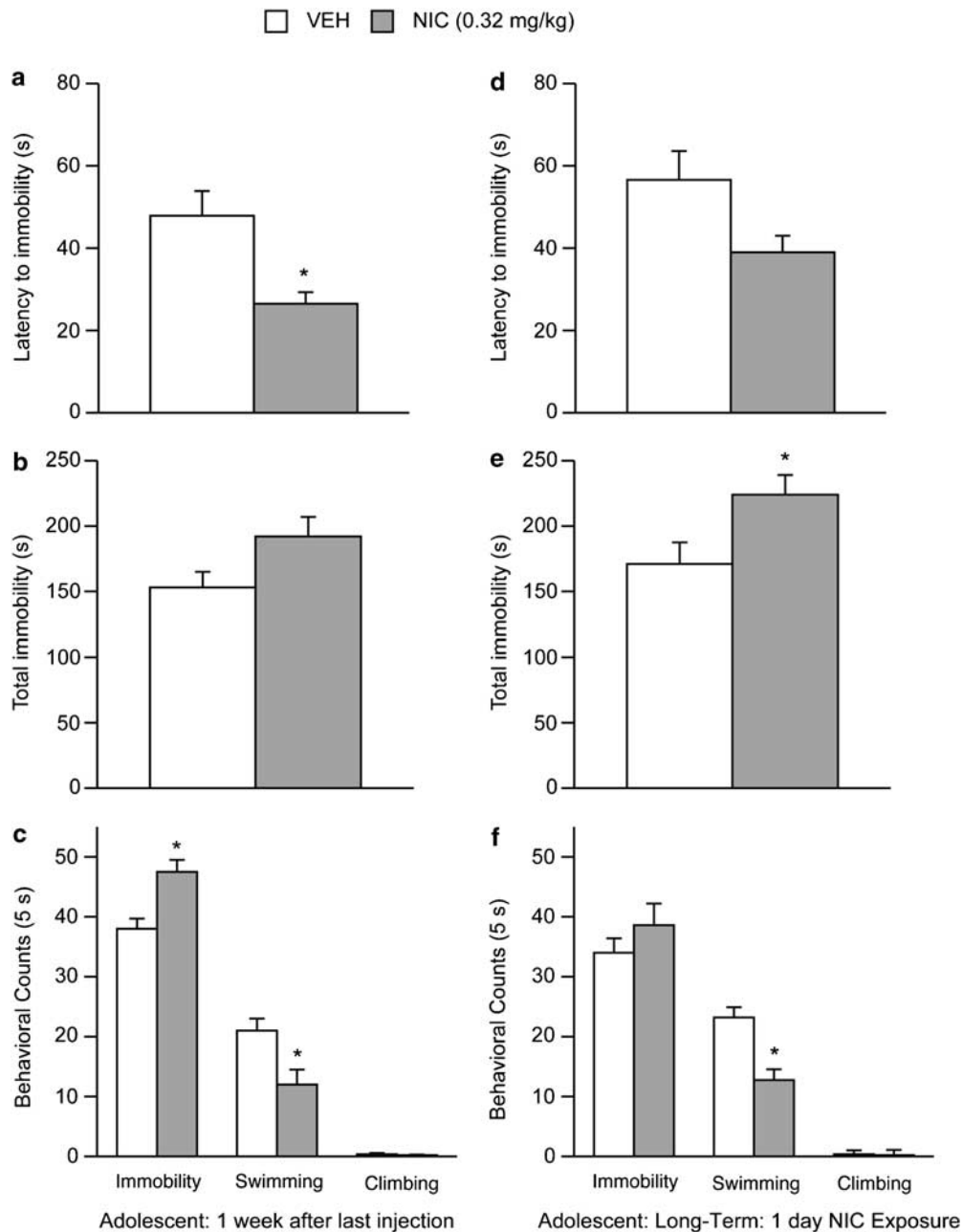
Figure 7 Effects of (−)-nicotine hydrogen tartrate (NIC) exposure in adolescent rats: 1 week after chronic exposure (a–c), and 1 month after a single day of exposure (d–f) on behavioral responsivity in the forced swim test (FST). After 1 week ($n = 18$): (a) NIC resulted in a significant decrease in latency to immobility of day 2 of the FST (*$p < 0.05$: different from VEH). (c) These rats show significantly higher immobility counts (*$p < 0.05$) and lower swimming counts (*$p < 0.05$) when compared to controls. 1 month after a single day of NIC, exposure ($n = 18$): (d) these rats show no statistical significant changes in latency to immobility, (e) but demonstrate significantly higher levels of total immobility (*$p < 0.05$), and (f) lower swimming counts (*$p < 0.05$) when compared to controls. Data are presented as latencies to become immobile, total immobility (in seconds), or swimming counts (mean ± SEM).

thus avoiding potential generalized motor activity from influencing the results from the FST. A pattern consistent with the various treatments was observed when assessing total immobility (Figure 8b), as this behavior was also influenced by adolescent and adult treatment (pretreatment × posttreatment interaction: $F_{(1, 42)} = 8.79$; $p < 0.005$). For instance, rats in the NIC/SAL group had significantly higher total immobility than the VEH/SAL ($p < 0.05$), VEH/NIC ($p < 0.05$), NIC/NIC ($p < 0.05$), NIC/FLX ($p < 0.05$), or the NIC/BPN ($p < 0.05$) groups,

respectively. NIC exposure also influenced immobility, swimming, and climbing ($F_{(1, 42)} = 10.95$; $p < 0.002$) counts (Figure 8c). More specifically, the NIC/SAL group had significantly higher levels of immobility ($p < 0.05$), and lower levels of swimming ($p < 0.05$) and climbing ($p < 0.05$) counts, whereas the VEH/NIC, NIC/NIC, NIC/FLX, and NIC/BPN had lower levels of immobility, and higher levels of swimming counts, respectively ($p < 0.05$). Higher levels of climbing were observed in all groups as compared to the NIC/SAL condition ($p < 0.05$).
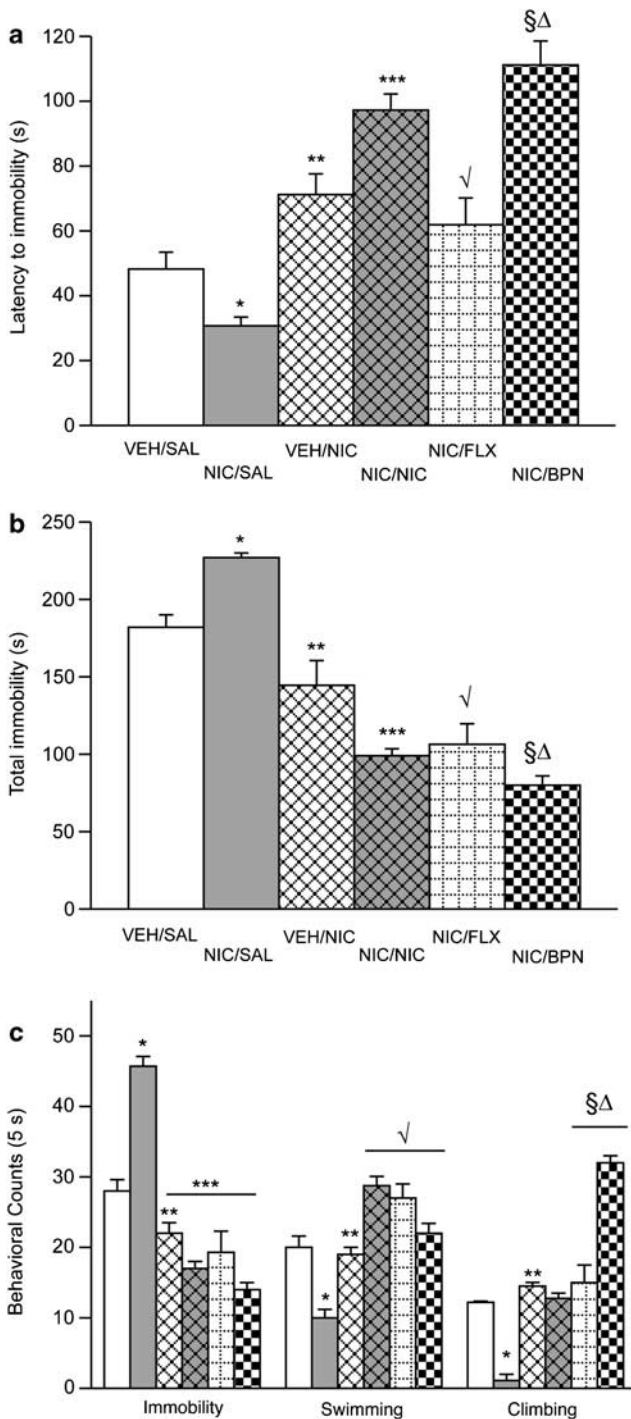
Figure 8 (−)-Nicotine hydrogen tartrate (NIC), FLX, and BPN reverse NIC-induced behavioral deficits on behavioral responsivity in the forced swim test (FST) (a–c). (a) NIC significantly decreased latency to immobility (*$p < 0.05$ vs VEH/SAL). NIC (***$p < 0.05$) and BPN ($^{\S}p < 0.05$) significantly increased latency to immobility when compared to the VEH/SAL and NIC/SAL groups (**$p < 0.05$ vs NIC/SAL; $\sqrt{}p < 0.05$ vs NIC/SAL; $^{\S\Delta}p < 0.05$ vs VEH/NIC and NIC/FLX). (b) Complementary pattern of results as measured by total immobility, as well as (c) immobility (**$p < 0.05$ vs NIC/SAL; ***$p < 0.05$ vs NIC/SAL), swimming (*$p < 0.05$ vs VEH/SAL; **$p < 0.05$ vs NIC/FLX; $\sqrt{}p < 0.05$ vs NIC/FLX), and climbing counts (*$p < 0.05$ vs VEH/SAL; **$p < 0.05$ vs NIC/SAL; $^{\S\Delta}p < 0.05$ vs all other conditions; $n = 48$). Data are presented as latencies to become immobile, total immobility (in seconds), or swimming counts (mean ± SEM).

Discussion

Given the relationship between nicotine dependence and mood disorders and that smoking often begins in adolescence, this study assessed the short- and long-lasting effects of nicotine exposure on behavioral reactivity to various emotion-eliciting stimuli in male rats. Our results demonstrate that exposure to nicotine during adolescence—but not during adulthood—leads to a depression-like state manifested in decreased sensitivity to natural reward, and enhanced sensitivity to stress- and anxiety-eliciting situations later in life. Our data show that behavioral dysregulation can emerge after 1 week of nicotine cessation, and that a single day of nicotine exposure during adolescence can be sufficient to precipitate a depression-like state in adulthood. Furthermore, we demonstrate that these nicotine-induced deficits can be rescued by subsequent nicotine or antidepressant treatment in adulthood. Together, these data suggest that nicotine exposure during adolescence results in a negative emotional state characterized by anhedonia (an inability to experience naturally rewarding and exciting stimuli), and heightened sensitivity to the adverse effects of stress.

Exposure to nicotine during adolescence resulted in significant decreases in rats' normal sensitivity to the rewarding properties of sucrose (a natural reward) in adulthood. Deficits in sucrose preference were not present in adolescents tested 24 h following nicotine, nor in rats exposed as adults. Although nicotine significantly decreased adolescents' bodyweight during treatment and could have influenced sucrose consumption, this is not likely the case because bodyweight between the groups did not differ when tested in adulthood (Figure 1a, inset). In addition, if bodyweight had influenced consumption, this would have resulted in decreased sucrose preference in the short-term adolescent group because their bodyweight was significantly lower than controls. Thus, the decrease in sucrose preference is likely due to nicotine's ability to alter responsiveness to the rewarding effects of the solution, because the overall liquid intake during testing did not differ between the groups. Brain reward pathways, such as the NAc and its dopaminergic input from the VTA, are crucial in regulating responses to both natural and drug rewards (Kelley and Berridge, 2002; Koob and Le Moal, 2008). Exposure to sweet solutions activates this system increasing dopamine in the NAc, whereas disruptions in the functioning of these pathways decrease preference for sucrose and other reward-related stimuli (Hajnal and Norgren, 2001; Kelley and Berridge, 2002; Shimura *et al*, 2002). These results are consistent with others indicating that exposure to nicotine, as well as other psychostimulants such as methylphenidate early in life, leads to long-lasting neural alterations resulting in decreased interest in natural rewards (ie anhedonia) in adulthood (Bolaños *et al*, 2003, 2008; Franke *et al*, 2008; Mague *et al*, 2005). It must be noted, however, that the effect size of nicotine on sucrose preference, though statistically significant, is small, and that nicotine did not influence total sucrose intake (Figure 4c, right panel). In addition, others have not detected differences in sucrose preference after adolescent exposure to nicotine (Slawecki and Ehlers, 2002). In the previous study, the authors administered nicotine (7.0 mg/kg per

day) through transdermal patches for a relatively short period (5 days), and tested rats on a 2% sucrose solution, whereas in this study we used a longer treatment regimen (15 days) that included twice-daily injections of 0.32 mg/kg nicotine, and tested with a 1% sucrose concentration. In addition, we exposed rats between PD 30 and 44, whereas in the previous study rats were exposed to nicotine between PD 35 and 40. Thus, it is conceivable that differences in treatment duration and drug dosage, as well as sucrose concentration, may account for the discrepancies between the studies. Alternatively, other physiological factors such as changes in metabolism, taste sensation, and kidney function induced by nicotine exposure during adolescence as potential mediators influencing sucrose consumption cannot be ruled out.

Our findings also indicate that early life nicotine enhances rats' sensitivity to aversive situations later in adulthood. More specifically, adult rats exposed to nicotine during adolescence exhibited enhanced reactivity to anxiogenic stimuli, as they spend significantly less time in the 'open arms' of the EPM, and more time engaged in self-grooming behavior, a well-known behavioral response to anxiety-eliciting situations (Spruijt et al, 1988). In addition, there were no differences in reactivity of adult rats exposed to the same regimen of nicotine exposure and testing in the EPM. These results are supported by demonstrations that nicotine exposure before adulthood results in higher indices of anxiety-like behaviors later in life (Huang et al, 2007; Slawecki et al, 2003, 2005). Under the appropriate conditions, behavioral reactivity in the OFT can also be used as an index of increased anxiety (Britton and Britton, 1981), thus it must be noted that the behavioral performance observed in the OFT under our experimental conditions do not compliment our findings of increased anxiety-like behaviors as measured in the EPM. Further studies with the FST revealed that adolescent nicotine exposure decreased latency to immobility and other escape-like behaviors, while increasing total immobility in adulthood, effects opposite to that of antidepressant treatments (Cryan et al, 2002; Lucki, 1997; Porsolt et al, 1977). These effects are not due to nicotine-induced deficits in motor activity because rats treated with nicotine (0.32 mg/kg) show no difference in distance traveled in the open field (Figure 2b). Our findings are supported by studies showing that exposure to nicotine and other psychostimulants results in depression-like states (Barr et al, 2002; Bolaños et al, 2003; Carlezon et al, 2003; Mannucci et al, 2006; Picciotto et al, 2002). Moreover, we show that these nicotine-induced behavioral deficits in the FST can be rescued by subsequent chronic exposure to the antidepressants FLX and BPN, or acute nicotine. These observations are in agreement with basic and clinical studies showing that nicotine has antidepressant properties (Salin-Pascual et al, 1996; Suemaru et al, 2006; Tizabi et al, 1999; Vazquez-Palacios et al, 2004), the well-known effects of antidepressant treatment in the FST (Cryan et al, 2002), and with studies suggesting that antidepressants can be effective in ameliorating dysphoric states followed by nicotine withdrawal/smoking cessation (Cryan et al, 2003; Hatsukami et al, 2008; Schnoll and Lerman, 2006). The cellular mechanism(s) underlying these effects are unknown. Nicotine, as well as FLX and BPN, interacts in a complex manner with serotonergic and dopaminergic systems by increasing neural activity and regulating neurotransmitter content to influence behavioral responding (Seth et al, 2002; Suemaru et al, 2006). Given that depression is associated with impaired functioning of these systems (Krishnan and Nestler, 2008b; Manji et al, 2001; Nestler and Carlezon, 2006), it is conceivable that these drugs enhance serotonin and dopamine transmission in mesolimbic brain regions to reverse depression-like behaviors induced by adolescent nicotine exposure.

Interestingly, nicotine did not influence behavioral responsivity of adolescent rats tested 24 h after cessation in any of the tests. Because signs of withdrawal can be observed up to 48 h following nicotine cessation in adult rodents (Kenny and Markou, 2001), we tested a separate group of adolescents in the FST 1 week after nicotine, and observed that these rats had shorter latencies to immobility and a lesser frequency of escape-like behaviors. These findings indicate that adolescent rats may not show the 'usual' trajectory of nicotine withdrawal as it is observed in adults (Wilmouth and Spear, 2006), or may reflect an age-dependent subsensitivity to the drug (Bolaños et al, 1998; Spear and Brake, 1983). We did not determine nicotine metabolism between the age groups in this study and thus it is also possible that our drug regimen and dose did not induce 'dependence' in adolescent rats, as studies assessing dysphoric aspects of nicotine withdrawal use higher doses than the one used here (Matta et al, 2007), and also because our treatment did not affect the adult rats. Nevertheless, our findings are in agreement with the notion that the full spectrum of effects after drug exposure during development may not appear until later in life (ie 'drug effects incubate'; Andersen and Navalta, 2004), and with recent epidemiological findings indicating that novice adolescent smokers show significantly longer latencies (ie weeks) to the onset of withdrawal from nicotine as compared to experienced smokers (Difranza and Ursprung, 2008; Fernando et al, 2006). Here we further show that a single day of nicotine exposure during adolescence can be sufficient to induce depression-like behaviors in adulthood. This finding may not be too surprising because occasional/limited nicotine use can be sufficient to induce dependence in adolescents (DiFranza, 2008; DiFranza et al, 2000), and because long-lasting synaptic changes can occur even after brief exposure to nicotine in young rats (Mansvelder and McGehee, 2000).

Together, our findings are in agreement with those showing that early-life exposure to psychostimulants has long-lasting effects: decreasing responsiveness to rewards, while enhancing sensitivity to aversive stimuli (Bolaños et al, 2003; Carlezon et al, 2003; Franke et al, 2008; Slawecki et al, 2003; Wiley et al, 2008). The mechanism(s) underlying nicotine's effects remain to be fully elucidated. Nicotine exposure has neurotoxic effects in several brain regions and neurotransmitter systems at distinct periods of development (Abreu-Villaca et al, 2003; Dwyer et al, 2008; Trauth et al, 2001). In addition, nicotine exposure regulates ΔFosB, CREB and ERK activity (Brunzell et al, 2003; Pluzarev and Pandey, 2004; Walters et al, 2005), factors associated with the regulation of mood and motivation (Bolaños and Nestler, 2004; Carlezon et al, 2005; Einat et al, 2003; Krishnan et al, 2008a; Wallace et al, 2008). Nicotine's actions on the nervous system are complex, and much more

detailed assessments of these phenomena, accounting for length of exposure, discontinuation, and developmental periods, are clearly needed (Adriani and Laviola, 2004; Barron et al, 2005; Carlezon and Konradi, 2004).

Despite the lack of appropriate animal models to mirror the human condition (Fisch, 2007; Nestler et al, 2002), our findings may have clinical implications. Nicotine can alleviate negative affect, and because depression, increased stress, and anxiety often emerge during adolescence (Andersen and Teicher, 2008; Spear, 2000), afflicted individuals may initiate smoking to regulate mood through a self-medication process (Breslau et al, 2004b; Markou et al, 1998). Consequently, adolescence represents a period of increased vulnerability because early life tobacco use is associated with higher risks for psychiatric disorders later in life, and early psychiatric-related problems are linked to greater tobacco use and dependence (Patton et al, 2006; Upadhyaya et al, 2003; Volkow, 2004). Thus, our findings that nicotine exposure during adolescence leads to dysregulated mood in rats parallel reports suggesting that cigarette smoking during adolescence leads to the onset of depression and other psychiatric disorders (Breslau et al, 2004a; Choi et al, 1997; Goodman and Capitman, 2000; Steuber and Danner, 2006; Windle and Windle, 2001; Wu and Anthony, 1999). It is thus conceivable that some adolescents may initiate smoking as a way of self-medicating preexisting mood disorder conditions (Alvarado and Breslau, 2005; Breslau et al, 2004b; Windle and Windle, 2001), whereas others would continue to smoke to manage mood dysregulation induced by their recreational exposure to nicotine itself (DiFranza et al, 2004; Parrott, 1999).

To summarize, our study shows that nicotine exposure during adolescence induces a negative emotional state characterized by anhedonia and heightened sensitivity to the adverse effects of stress later in life, that subsequent nicotine or antidepressant treatment normalize these depression-like behaviors, and that a relatively brief exposure to the drug can have long-lasting negative consequences (DiFranza, 2008; Mansvelder and McGehee, 2000). Within this context, our findings, together with those indicating that nicotine exposure during adolescence enhances risk for addiction (Adriani et al, 2003, 2006; McQuown et al, 2007), could serve as a potential model of comorbidity. Moreover, our findings underscore the need for further assessment of long-term neurobiological adaptations induced by early life experiences contributing to the pathophysiology of neuropsychiatric disorders later in life.

DISCLOSURE/CONFLICT OF INTEREST

The authors have no financial or competing interests to declare.

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