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Long-term changes in fear conditioning and anxiety-like behavior following nicotine exposure in adult versus adolescent rats

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Abstract

Adolescent nicotine exposure is associated with long-term use, and it has been suggested that this vulnerability to addiction may relate to lasting anxiogenic effects of the drug. However, few studies have addressed long-term effects of adolescent nicotine, and fewer yet have compared adolescent to adult exposure. Male and female Long-Evans rats continuously received nicotine bitartrate or sodium tartrate via osmotic mini-pumps over 15 days either during adolescence (p28–42) or adulthood (p85–99). Initial nicotine dose (free base) was either low (1 mg/kg/day) or high (2 mg/kg/day). Open field behavior and fear conditioning were assessed in adulthood, 1 month post-dosing. Animals pretreated with nicotine during adolescence showed less center time in a novel open field than sham controls. Conversely, the two nicotine doses differentially affected fear conditioning. Animals pretreated with low nicotine during adolescence demonstrated superior acquisition of the task compared to sham control animals; however, unlike either high nicotine-pretreated or sham control animals, they failed to extinguish the learned behavior. In contrast, animals pretreated during adulthood did not behave significantly different from sham controls on either task. Overall, nicotine-pretreatment during adolescence induced effects on behaviors related to fear and anxiety in adulthood, while comparable pretreatment during adulthood failed to produce significant residual effects.

Keywords: Adolescence; Addiction; Open field; Fear conditioning

1. Introduction

Attention in addiction literature has turned recently to age-dependent effects, specifically the potential for long-term effects following adolescent exposure to drugs of abuse. Interest in the effects of nicotine use during adolescence is particularly pertinent, given that over one-third of high school students in the U.S. are reported to smoke (National Institutes of Health, 2001). The suggestion that smoking initially in adolescence may confer (or denote an existing) risk for addiction is supported by many observations, including that roughly 90% of adult smokers began smoking before the age of 21 (American Lung Association, 2003). Additionally, increased daily cigarette consumption and decreased ability to quit are correlated with use beginning in early adolescence (Chen and Millar, 1998).

Indeed, several studies have reported greater sensitivity to nicotine's effects in adolescent animals, compared to adult animals, when tested during drug administration (Elliott et al., 2004; Rezvani and Levin, 2004; Schochet et al., 2004; Trauth et al., 2000). There is also evidence that adolescents may be particularly susceptible to nicotine dependence, including that adolescent rats appear to perceive low doses of nicotine as rewarding, while adults do not (Vastola et al., 2002), and adolescents seem to be subject to less severe somatic withdrawal effects (O'Dell et al., 2006). While these studies are certainly relevant, perhaps more interesting is the question of whether age-dependent effects are long lasting.

In fact, there are several reports of lasting behavioral and molecular effects of nicotine exposure specific to the adolescent period, measurable weeks and even months following the last dose. Among these are upregulation of nicotinic cholinergic

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receptors in the brain (Trauth et al., 1999), increased propensity to self-administer nicotine in adulthood (Adriani et al., 2003), and decreased reward associated with cocaine in adulthood (Kelley and Middaugh, 1999). These studies suggest there are features unique to the adolescent brain, possibly related to increased plasticity, which make it particularly vulnerable to nicotine's neurobehavioral effects.

It has also been suggested that nicotine's long-term addictive effects on adolescents may relate to emotional behaviors, such as anxiety and depression (DiFranza et al., 2004; Slawecki et al., 2005; Torrella et al., 2004), and several rodent studies have focused on such long-term emotional effects. For example, mice that received nicotine during mid-adolescence showed decreased exploration and activity compared to controls when tested in adulthood (Adriani et al., 2004), consistent with an anxiogenic and depressive profile. In addition, open field studies have shown anxiety-like behavior in adult male Sprague-Dawley rats treated with nicotine during adolescence, evidenced by reduced locomotor activity (Slawecki and Ehlers, 2002; Slawecki et al., 2003), and at least one study reported this effect to be specific to females (Trauth et al., 2000).

Conversely, hyperactivity in the open field has been reported in adult male (Faraday et al., 2001, 2003a), but not female (Elliott et al., 2005; Faraday et al., 2001), Sprague-Dawley rats chronically treated with nicotine in adolescence; however, this discrepancy may be due to the fact that these studies housed animals under a reverse light/dark cycle and delivered much larger nicotine doses. It is also noted that differing lengths of locomotor sessions across studies may contribute to discrepancies in results. Decreased food-oriented behavior following adolescent exposure to nicotine in Sprague-Dawley rats has also been reported (Slawecki et al., 2003) and is indicative of lasting anxiety-like behavior.

However, to date, no studies investigating the long-term effects of adolescent nicotine exposure on emotional learning (e.g., fear conditioning) have been published. In the current study we examined the effect of chronic adolescent nicotine exposure on locomotor activity as well as on fear-related learning in the rat. We hypothesized that adolescent nicotine exposure would produce anxiety-like behavior in a novel open field and increased performance in the fear conditioning task when assessed in adulthood.

In addition, given the evidence that rat strain and sex influence response to nicotine, these parameters should be taken into account when considering behavioral outcomes. Studies focusing on strain differences suggest that variation with respect to this parameter may offer a model of individual human differences in nicotine-induced alteration of behavior (Faraday et al., 1999, 2003b, 2005). For example, the Long-Evans strain is reported to develop tolerance to some of nicotine's effects more quickly than the Sprague-Dawley strain and has been recommended as an animal model of humans particularly vulnerable to nicotine dependence (Faraday et al., 1999). In the present study, we characterized the neurobehavioral response of the Long Evans rat to adolescent administration of nicotine. Additionally, since male and female responses have been shown to vary in previous reports, both sexes are examined here.

2. Materials and methods

2.1. Animals and drugs

Male and female Long-Evans rats (Harlan, Indianapolis, IN, USA) continuously received nicotine bitartrate or sodium tartrate (sham control) dissolved in 0.9% saline via subcutaneously implanted Alzet osmotic pumps (Durect Corp., Cupertino, CA, USA). Following anesthesia with equithesin in female (3.0 mg/kg) and male (3.5 mg/kg) animals, the lower back was shaved and a small incision made to permit implantation. A single 14-day pump (Model 1002) was implanted either during adolescence (p28–42) or adulthood (p85–99). These groups are referred to adolescent- and adult-pretreatment groups for clarity, since testing actually took place during adulthood for both.

Initial nicotine dose (free base) was either low (1 mg/kg/day) or high (2 mg/kg/day). Both of these doses can be expected to produce nicotine plasma levels lower than that of a heavy smoker (Slotkin, 2002). Pumps were removed on the 15th day following implantation. Despite being marketed as a 14-day pump, infusion was continuous until removal, as this model does not become exhausted until at least Day 18 (calculations based on manufacturer information). Nicotine dose rates diminished over time according to weight gain; final rates (free base) in mg/kg/day are as follows: low nicotine dose (adolescent male, 0.48; adolescent female, 0.51; adult male, 0.87; adult female, 0.84), high nicotine dose (adolescent male, 0.95; adolescent female, 1.03; adult male, 1.74; adult female, 1.68).

Sample size breakdown for animals pretreated during adulthood is as follows (male and female numbers are indicated): control, $n=20$ (11 m, 9 f); low nicotine, $n=17$ (7 m, 10 f); high nicotine, $n=19$ (10 m, 9 f). Sample sizes for dose groups pretreated during adolescence varied slightly between the two tasks due to data loss during testing and are as follows: *open field*: control, $n=22$ (9 m, 13 f); low nicotine, $n=23$ (11 m, 12 f); high nicotine, $n=20$ (8 m, 12 f); *fear conditioning*: control, $n=22$ (10 m, 12 f); low nicotine, $n=21$ (10 m, 11 f); high nicotine, $n=19$ (8 m, 11 f).

Animals were housed individually on a 12 h light/12 h dark cycle (lights on at 07:00) with *ad libitum* access to food and water and were handled regularly during both the drug exposure and abstinence period to minimize handling stress during behavioral testing. All behavioral testing took place during the light phase. All animal experiments were approved by the University Institutional Animal Care and Use Committee.

2.2. Behavioral testing

Locomotor behavior in a novel open field and fear-conditioning/extinction were assessed approximately 1 month following the end of dosing. Animals received one 15-min trial on a single day of testing in the open field apparatus ($42\times42\times30$ cm^3). Center (17.8×17.8 cm^2) time duration and total distance traveled were measured (Viewpoint, Montreal, QC, Canada). A solution of 95% EtOH was used between animals to clean the apparatus. Behavioral scores were averaged in three 5-min intervals.

The day after open field testing, animals were trained in a fear-conditioning task. Fear conditioning and extinction took place in a transparent ($26 \times 26 \times 18$ cm^3) Plexiglas chamber (San Diego Instruments, San Diego, CA, USA) using a PC laptop equipped with SDI Freeze Monitor System for Windows®, Version 1.4.4. The chamber floor consisted of 16 stainless steel bars connected to a shock scrambler, through which the unconditioned stimulus (US) was delivered. The US consisted of 2 s of 0.5 mA scrambled foot shock. The conditioned stimulus (CS) was a white noise tone (80 dB), which lasted 20 s and was delivered through a speaker in the chamber lid. Rats were introduced to the chamber via the top of the apparatus. The same chamber was used for cue-dependent conditioning/extinction, but steps were taken to alter the perceived environment. These steps included placing an opaque piece of Plexiglas over the floor bars, lowering the ambient light level in the chamber room, and introducing a foreign odor (Caribbean Cooler Renuzit® Adjustable Cone air freshener). The chamber was cleaned with 95% EtOH between animals.

Training on Day 1 consisted of three CS tones presented at 160, 220 and 280 s, each of which were 20 s in length, and overlapped and co-terminated with the US shock. No shocks were administered after Day 1. Contextual conditioning was tested on Day 2, for which animals were returned to the same environment without the CS. Cued conditioning was tested on Day 3 during which the environment was altered as described above and the CS tone was presented in the same pattern as Day 1. Cued extinction (termination of the freezing behavior) was tested on Day 9 and was conducted in the same manner as Day 3. Movement in the chamber was detected via a photo-beam array. The dependent measure (latency to movement) was operationally defined as time elapsed to three consecutive beam breaks within a 10-s block of time and was considered to reflect freezing. 10-s blocks of time were averaged together for each minute of the 6-min daily testing sessions.

2.3. Data analysis

Repeated measures ANOVAs were conducted for open field measures across the three averaged time points (1st, 2nd, and 3rd 5-min block of 15-min trial). For fear conditioning/extinction, repeated measures ANOVAs were conducted on the last 3 min of each day (following each cue presentation), except for contextual conditioning. When appropriate, Tukey's HSD post hoc analyses were performed. Significance was assumed at $p < 0.05$. Means are reported ± S.E.M.

3. Results

Initially, the factors of Pretreatment Age (adolescent or adult), Dose (control, low, or high nicotine), and Sex were included in all statistical analyses. However, a significant main effect or interaction involving sex was only seen for total distance traveled in the open field; therefore, with the exception of this measure, the reported analyses include only the factors of Pretreatment Age and Dose.

3.1. Novel open field

Statistical analysis of time spent in the center of the novel open field revealed a significant Pretreatment Age × Dose interaction ($F_{2,115} = 5.56$, $p < .01$). Follow-up analysis for the adolescent-pretreatment group showed a simple main effect of dose ($F_{2,62} = 3.81$, $p < .05$; see Fig. 1). Post hoc analyses for the adolescent-pretreatment group showed the high nicotine group spent significantly less time in the center portion of the arena than the sham control group ($p < .05$). Univariate analyses for each time point showed significance only on the average of the last 5 min of the single open field trial ($p < .05$); animals pretreated with high nicotine during adolescence spent less time in the center of the field than sham control animals ($p < .05$). No dose effect was present for the adult pretreatment group. In addition, there was a significant main effect of pretreatment age ($F_{1,115} = 6.03$, $p < .05$), with adult-pretreated animals showing greater center duration than adolescent-pretreated animals.

As mentioned, total distance traveled in the novel open field was the only measure to show significant sex effects and therefore, the factor was kept in the analysis. There was a significant main effect of sex ($F_{1,109} = 4.19$, $p < .05$), with females traveling

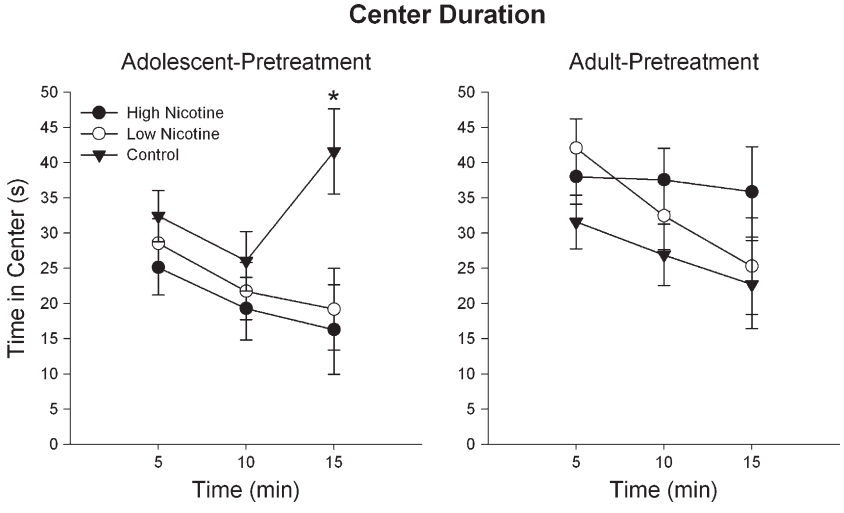

Fig. 1. Time spent in the center of the open field is shown according to age of pretreatment. Rats received a single 15-min trial; 5-min averaged blocks of the trial are shown. Significant univariate analyses are indicated (*$p < .05$).

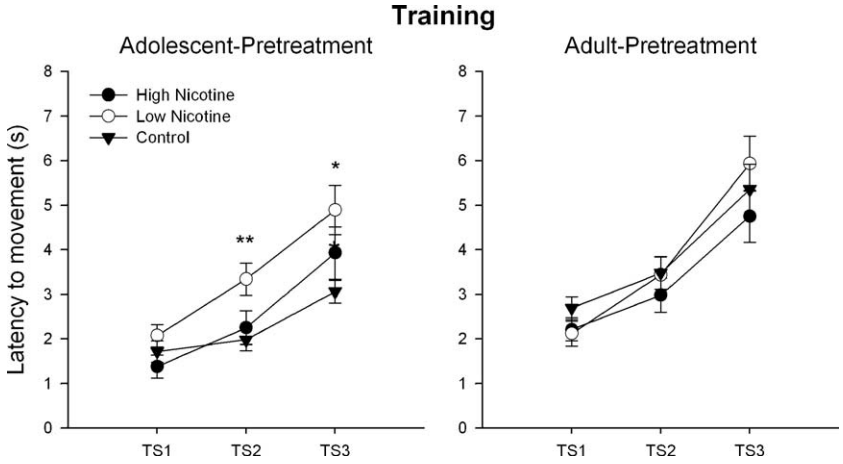

Fig. 2. Data for the Training Day (Day 1) of fear conditioning is shown according to age of pretreatment. Latency to movement following tone-shock pairings (TS) for the last 3 min of testing is represented. Significant univariate analyses are indicated (*$p<.05$; **$p<.01$).

farther than males. There was also a significant main effect of pretreatment age on total distance traveled ($F_{1,109}=51.98$, $p<.001$), as expected, with adolescent-pretreated animals traveling greater overall distances than adult-pretreated animals. Importantly, however, there were no significant interactions involving dose group, nor was there a significant main effect of dose group on total distance traveled, indicating that the effect of dose on center time above was not due to differences in activity levels.

3.2. Fear conditioning

As mentioned, all fear conditioning days involving the CS (cue) (i.e., Days 1, 3, and 9) will be discussed in terms of the last 3 min, i.e., the time period encompassing cue presentation. No significant main effects or interactions involving sex were seen for any of the fear conditioning measures, so reported results include only the factors of Pretreatment Age and Dose.

3.2.1. Training

On Day 1, a significant main effect of dose was seen ($F_{2,112}=3.51$, $p<.05$) across pretreatment age. Post hoc tests showed the low nicotine group to be significantly different from the high nicotine group ($p<.05$). When age of pretreatment was considered, the main dose effect remained significant for the adolescent-pretreatment group ($F_{2,59}=4.98$, $p<.05$), but not the adult-pretreatment group. Adolescent-pretreated animals given low nicotine exhibited greater freezing behavior in adulthood than sham control animals ($p<.05$). When univariate analyses were performed for each minute, a significant effect of dose was seen for the second tone-shock pairing (TS2; $p<.01$); the adolescent-pretreatment low nicotine group showed significantly more freezing than the sham control ($p<.01$) and high nicotine groups ($p<.05$). The adolescent-pretreatment low nicotine group also showed more freezing than the sham control group on the third tone-shock pairing (TS3; $p<.05$). In addition, a significant main effect for pretreatment age was seen ($F_{1,112}=15.78$, $p<.001$), with the adult-pretreatment group showing more freezing than the adolescent-pretreatment group. Training graphs are shown in Fig. 2.

3.2.2. Contextual conditioning

The only significant difference seen on Day 2 was a main effect for pretreatment age ($F_{1,108}=45.68$, $p<.001$; data not

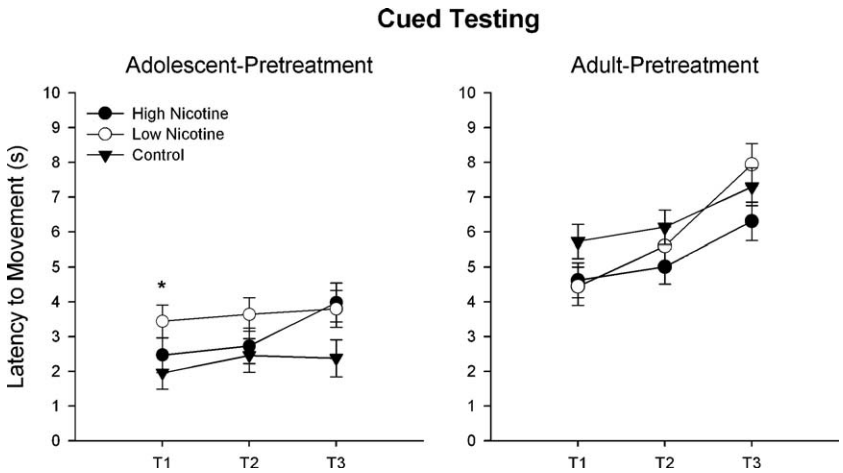

Fig. 3. Data for the Cued Conditioning Day (Day 3) of fear conditioning is shown according to age of pretreatment. Latency to movement following each tone presentation (T) for the last 3 min of testing is represented. Significant univariate analyses are indicated (*$p<.05$).

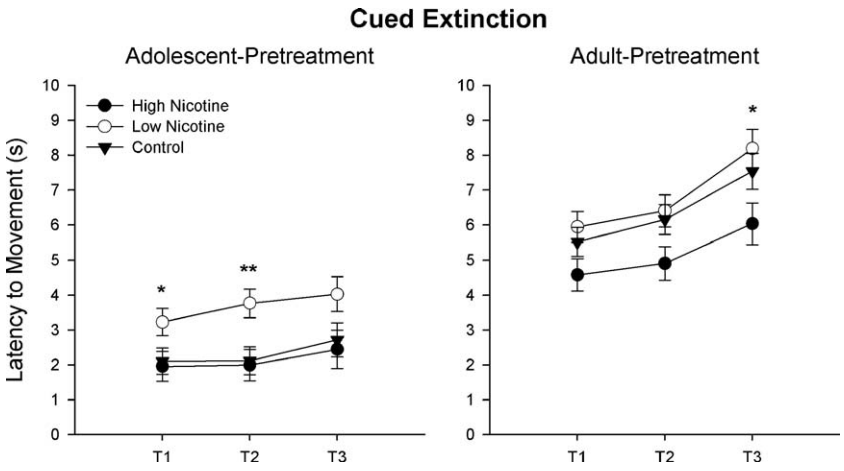

Fig. 4. Averaged data for the Cued Extinction Day (Day 9) of fear conditioning is shown according to pretreatment age. Latency to movement following each tone presentation (T) for the last 3 min of testing is represented. Significant univariate analyses are indicated (*$p<.05$; **$p<.01$).

shown), with adult-pretreated animals (3.74±0.21) showing more freezing behavior than adolescent-pretreated animals (1.83±0.20).

3.2.3. Cued conditioning

On Day 3, there was a significant Pretreatment Age×Dose interaction ($F_{2,110}=3.22$, $p<.05$) that, when further investigated, revealed a significant simple main effect of dose for the adolescent-pretreatment group only ($F_{2,58}=3.43$, $p<.05$). Post hoc analysis showed the animals pretreated with low nicotine during adolescence spent significantly more time freezing than sham control animals ($p<.05$). Univariate analyses by minute showed the adolescent-pretreated low nicotine group to freeze more than the sham control group at the first tone (T1; $p<.05$) only. On Day 3, there was also a significant main effect of pretreatment age ($F_{1,110}=70.71$, $p<.001$), again with adult-pretreated animals showing greater freezing than adolescent-pretreated animals. In addition, there was a significant Pretreatment Age×Minute interaction ($F_{1.79,196.7}=7.44$, $p<.01$); univariate analyses on minutes 4, 5, and 6 on Day 3 showed the adult-pretreatment group froze significantly more than the adolescent-pretreatment group on each ($p<.05$). Cued conditioning graphs are shown in Fig. 3.

3.2.4. Cued extinction

Day 9 analyses showed significant main effects for both pretreatment age ($F_{1,107}=119.87$, $p<.001$) and dose group ($F_{2,107}=8.71$, $p<.001$). The adult-pretreatment group again showed more freezing than the adolescent-pretreatment group. Post hoc follow-up of the main dose effect showed the low nicotine group to freeze significantly more than either the sham control ($p<.05$) or high nicotine ($p<.001$) groups.

When pretreatment age groups were viewed separately, there were significant dose effects for both the adolescent-pretreatment ($F_{2,58}=4.67$, $p<.05$) and adult-pretreatment groups ($F_{2,49}=5.42$, $p<.05$). Post hoc analyses for the adolescent-pretreatment group revealed low nicotine animals showed more freezing than either sham control ($p<.05$) or high nicotine animals ($p<.05$). Univariate analyses for each minute showed a significant effect of dose on T1 ($p<.05$) and on T2 ($p<.01$). There was a trend for animals pretreated in adolescence with low nicotine to show more freezing than sham control ($p=.07$) and high nicotine animals ($p=.06$) on T1. On T2, adolescent-pretreated low nicotine animals froze significantly more than sham control ($p<.05$) and high nicotine animals ($p<.01$).

Post hoc analyses for the adult-pretreatment group only showed that the low dose animals froze more than high nicotine animals ($p<.01$). Univariate analyses for each minute showed a significant effect of dose on T3 only ($p<.05$). On T3, adult-pretreated low nicotine animals froze significantly more than high nicotine animals ($p<.05$). Therefore, unlike adolescent-pretreated animals, neither of the adult-pretreatment nicotine groups differed significantly from the sham control group. Cued extinction graphs are shown in Fig. 4.

4. Discussion

We demonstrate that early adolescent exposure to nicotine in rats produces anxiety-like behaviors that are detectable in adulthood, while comparable nicotine exposure in adult rats fails to produce such effects. The open field data presented here are in agreement with previous studies using similar nicotine doses, administration methods, and light/dark schedules (Slawecki and Ehlers, 2002; Slawecki et al., 2003). However, our study is unique in that it included an adult exposure group, which received the same duration of abstinence, showing that lasting effects in open field behavior are specific to exposure during adolescence. Taken together with our results, these studies suggest a lasting anxiogenic profile following adolescent exposure to nicotine.

We extend the available data on long-term emotional effects following adolescent nicotine administration by examining fear learning. Unlike the open field data, the fear conditioning/extinction results show a somewhat divergent effect for animals pretreated in adolescence with different nicotine doses; this effect resembles earlier data from our lab on the long-term effects of adolescent nicotine on spatial learning (McDonald et al., 2003). In both experiments, adolescent-pretreated low nicotine animals demonstrated superior acquisition of the behavioral task

compared to high nicotine animals (and compared to control animals in the present study). These results imply long-term improved learning ability in adolescent rats exposed specifically to low levels of nicotine.

However, it is important to note that superior learning ability may be maladaptive in emotional contexts involving fear. Our data show that while adult animals chronically exposed to a low dose of nicotine during adolescence show superior acquisition of the task, they fail to show normal extinction of the freezing behavior. Interestingly, failure to extinguish following fear learning has been used to model anxiety disorders (Likhtik et al., 2005). It may be argued that since the low dose nicotine animals acquired the task more robustly than control animals, they should not be expected to extinguish at the same rate; however, superior learners in other studies have demonstrated extinction at a faster rate (Tang et al., 1999), adding to the evidence that learning and extinction are mediated by separate processes (for a review, see Kim and Jung, 2006).

Recent assessments of the neural bases of extinction indicate the involvement of the frontal cortex (Kim and Jung, 2006) and cholinergic systems (Izaki et al., 2001). Specifically, the nicotinic cholinergic antagonist, mecamylamine, has been shown to disrupt extinction when infused directly into prefrontal cortex (Maruki et al., 2003). These reports suggest impairment of extinction when nicotinic receptor function in frontal cortex is interrupted. It is potentially of relevance that our lab has found changes in mRNA levels of the alpha 4 subunit of the nicotinic cholinergic receptor in frontal cortex following adolescent-pretreatment with low nicotine (Stanton et al., 2005). Specifically, rats that received a chronic low dose of nicotine, comparable to that employed in the current study, during periadolescence (p22–69) showed significant decreases in frontal cortex alpha 4 subunit mRNA when examined in adulthood compared to sham control and high nicotine dose animals. We note that mRNA levels are not always indicative of protein expression, thus further investigation is needed; however, these findings suggest a possible molecular link between nicotine and anxiety-like behavior.

There was no clear evidence of extinction on Day 9 for the adult-pretreatment group in the current study. Thus, we cannot rule out the possibility that a ceiling effect in these animals occluded detection of potential differences between control and low nicotine animals in the fear conditioning task. An additional, delayed extinction trial might have helped to resolve this issue. Also, since adolescent animals may be more vulnerable to the effects of isolation than adults (Vanderschuren et al., 1997), use of single-housing in the current study may have affected specific outcomes. However, our results are in agreement with studies that used paired-housing, except for single housing during a 5-day dosing period (Slawecki and Ehlers, 2002; Slawecki et al., 2003), suggesting that housing does not have a significant impact.

It is interesting that sex does not seem to modulate nicotine's effects in our study, given that previous studies have reported sex effects. However, other studies looking at long-term effects of nicotine when administered in adolescence have used Sprague-Dawley rats, as opposed to Long-Evans, and there are reports that these two rat strains differ by sex in their responses to nicotine (Faraday et al., 1999, 2003b, 2005). Our study suggests that male and female Long-Evans rats behave similarly on novel open field and fear conditioning tasks in response to adult or adolescent nicotine administration (at these doses) followed by an abstinence period. Since most studies of this nature in rats have used the Sprague-Dawley strain, our study helps to characterize the response of the Long-Evans rat, and as mentioned previously, may better represent human adolescents with particular vulnerability to nicotine dependence.

Human adolescent nicotine use is a prominent societal issue, the effects of which are detectable in users into adulthood. Our data provide evidence that adolescents who chronically use nicotine become anxious adults in the absence of nicotine. Since human data suggest a common pattern of adolescent nicotine use that includes periods of cessation (Wellman et al., 2004), it is possible that the development of anxiety in the absence of nicotine contributes to dependence. Low severity of withdrawal symptoms in adolescents (O'Dell et al., 2006) likely contributes to this cycle as well. Moreover, we provide evidence that lower doses of chronic nicotine in adolescence enhance fear learning and impair fear extinction in adulthood, further supporting the development of an anxious emotional state. This is particularly disconcerting given that smoking fewer cigarettes per day is typically considered healthier than smoking a high amount, and studies indicate that, among adolescents who start smoking, 87% of them are considered sporadic or occasional smokers (Wellman et al., 2004).

As mentioned previously, since most adult smokers began using nicotine as teenagers, changes evident in behavior and neurobiology following adolescent nicotine exposure may provide insight into long-term addiction liability. Long-term effects of an emotional nature, including anxiety and depression, may be part of a cycle leading to recurrent use and addiction. Understanding emotional effects may give rise to interventions that can be used to break this cycle.

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