Original investigation

Chronic Nicotine Treatment During Adolescence Attenuates the Effects of Acute Nicotine in Adult Contextual Fear Learning

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Abstract

Introduction: Adolescent onset of nicotine abuse is correlated with worse chances at successful abstinence in adulthood. One reason for this may be due to enduring learning deficits resulting from nicotine use during adolescence. Previous work has indicated that chronic nicotine administration beginning in late adolescence (PND38) caused learning deficits in contextual fear when tested in adulthood. The purpose of this study was to determine if chronic nicotine treatment during adolescence would alter sensitivity to nicotine's cognitive enhancing properties in adulthood.

Methods: C57BL/6J mice received saline or chronic nicotine (12.6 mg/kg/day) during adolescence (postnatal day 38) or adulthood (postnatal day 54) for a period of 12 days. Following a 30-day protracted abstinence, mice received either an acute injection of saline or nicotine (0.045, 0.18, and 0.36 mg/kg) prior to training and testing a mouse model of contextual fear.

Results: It was found that chronic nicotine administration in adult mice did not alter sensitivity to acute nicotine following a protracted abstinence. In adolescent mice, chronic nicotine administration disrupted adult learning and decreased sensitivity to acute nicotine in adulthood as only the highest dose tested (0.36 mg/kg) was able to enhance contextual fear learning.

Conclusions: These results suggest that adolescent nicotine exposure impairs learning in adulthood, which could increase the risk for continued nicotine use in adulthood by requiring administration of higher doses of nicotine to reverse learning impairments caused by adolescent nicotine exposure.

Implications: Results from this study add to the growing body of literature suggesting chronic nicotine exposure during adolescence leads to impaired learning in adulthood and demonstrates that nicotine exposure during adolescence attenuates the cognitive enhancing effects of acute nicotine in adulthood, which suggests altered cholinergic function.

Introduction

Tobacco use is a leading cause of preventable death worldwide.¹ In the United States, it accounts for 480 000 deaths a year and an additional \$300 billion in related health care costs.² While most individuals who smoke want to quit, few have long-term success in remaining abstinent.³ One reason for the abysmal abstinence success may be due to tobacco use during adolescence. Previous research

has indicated that adolescent tobacco and nicotine use results in more severe dependence later in life and is associated with cognitive impairments in both clinical and rodent models.^{4–11} The persistent cognitive impairments that arise as a result of adolescent nicotine use may have profound effects on mental health. Changes in cognition are associated with several mental health disorders including depression,^{12,13} bipolar disorder,^{14,15} and schizophrenia.^{14,16,17} In addition,

several of these mental health disorders are also associated with high rates of smoking.¹⁸

Adolescence is a period of high susceptibility to substance use and abuse and represents a window of unique age-dependent responses to nicotine.^{19–21} Deficits caused by adolescent nicotine exposure are especially alarming when considering nicotine exposure during adolescence produces long-term learning deficits that persist into adulthood.^{7,8} For example, adult mice that received nicotine (12.6 mg/kg/day) for a period of 12 days during adolescence (PND38–50) and underwent a 30-day drug wash out period had deficits in contextual fear conditioning (hippocampus-dependent) but not cued fear conditioning (hippocampus-independent).^{22–24} Adult mice (PND53–65) that underwent similar treatment during adulthood did not display the learning deficit. Thus, adolescence represents a critical window for the induction of long-term cognitive impairments resulting from chronic nicotine use. These effects are not limited to animal models as clinical literature has demonstrated young smokers showed deficits in verbal working memory that worsen with 24 hours of nicotine abstinence.^{6,9} Sadly, recent reports suggest even passive exposure to nicotine during development through secondhand smoke correlates with deficits in attention and worse outcomes in mental health.¹⁰ While it is known that adolescent exposure to chronic nicotine results in long-term learning deficits, the underlying biological mechanism is unclear. One potential mechanism is altered acetylcholinergic function.^{25,26}

The objective of this study is to determine if adolescent nicotine exposure alters adult sensitivity to the effects of nicotine on learning, which would suggest altered cholinergic function. It has been shown in adult mice that multiple doses of acute nicotine will enhance contextual learning with an inverted-U dose-response pattern.^{25,26} Further, adult mice withdrawn from chronic nicotine did not demonstrate long-term changes in the sensitivity to subsequent nicotine administration.²⁵ Therefore, the purpose of this study is to determine if chronic nicotine exposure during adolescence shifts adult sensitivity to acute nicotine effects on learning. This is important because if sensitivity to the effects of nicotine on cognition is altered as a result of adolescent nicotine treatment, this may alter adult nicotine use and impact the development of nicotine addiction.

Methods

Subjects

Subjects were male C57BL/6J male mice that were non-littermates acquired from Jackson Laboratory (Bar Harbor, ME) and shipped to our facilities in Philadelphia, PA. Shipped animals arrived to our facility as adolescents or adults, at either postnatal day 31 (p31) or postnatal day 47 (p47), and were housed in pairs of 4. Chronic drug administration began at p38 (adolescence) or p54 (adulthood). These ages were chosen based on the commonly used age-range of adolescence occurring between p20 and p40 in rodents,²⁷ and previous work demonstrating chronic nicotine administration beginning at p38 caused learning deficits in adulthood following a protracted abstinence period.⁷ Implantation and removal of osmotic mini pumps that continuously deliver nicotine has consistently induced withdrawal related learning deficits in contextual fear^{7,28–30} (and raises plasma cotinine levels to that of human smokers).^{7,30} All animals received *ad libitum* access to food and water, and all procedures occurred between 10:00 AM and 5:00 PM during the light phase of a 12 hours light/dark cycle. Behavioral and surgical procedures were approved by the Temple University Institutional Animal Care and Use Committee.

Experimental Conditions

For adolescent and adult mice, the following groups were tested for contextual and cued fear learning 30 days following pump removal: (1) chronic saline and acute saline (WCS-Saline), (2) chronic saline and acute nicotine (WCS-Nicotine), (3) chronic nicotine and acute saline (WCN-Saline), and (4) chronic nicotine and acute nicotine (WCN-Nicotine). The WCS-Nicotine and WCN-Nicotine groups included a range of acute nicotine doses (0.045, 0.18, and 0.36 mg/kg).

Drug Preparation and Surgery

For all experimental groups, mice were treated chronically with nicotine or saline beginning at either p38 (adolescent) or p54 (adult). Drug administration occurred for 12 days and on the 12th day pumps were removed, described in detail below. Thirty days after pump removal (p80 or p96), mice were trained in fear conditioning and 24 hours later tested (p81 or p97). Nicotine tartrate salt (Sigma, St. Louis, MO) was dissolved in physiological saline and administered via subcutaneous osmotic mini pumps (Alzet #1002). Micro-osmotic pumps that administered 0.25 μ L/h of solution were filled with 100 μ L of either a nicotine solution (12.6 mg/kg/day freebase) or vehicle solution (0.09% saline) and inserted subcutaneously via intrascapular incision to the upper back of the mouse. This dose has been shown to produce cotinine levels comparable to human smokers³⁰ and also leads to long-term deficits in learning and memory when administered to adolescent mice.⁷ Twelve days after implantation, pumps were removed. All procedures were performed under sterile conditions with 2–3% of isoflurane as an anesthetic. Acute doses of nicotine (0.045, 0.18, and 0.36 mg/kg) were prepared by dissolving nicotine tartrate in physiological saline and administered intraperitoneally 5 minutes before mice were placed in the fear conditioning apparatus for training or testing. Acute doses were based on previous literature showing enhancement of contextual fear at the same dose and are reported as nicotine freebase.^{7,25}

Apparatus

Mice were trained and tested for contextual fear conditioning in four identical conditioning chambers (18.8 × 20 × 18.3 cm) that were housed within sound-attenuating boxes (MED Associates, Alban, VT). Each chamber consisted of Plexiglas front and rear walls and ceiling, with stainless-steel walls on the sides. The floor was made of metal rods that were connected to a shock generator and scrambler. Ventilation fans were mounted to the side to provide background noise, and a speaker mounted on the left wall was used to deliver a white noise conditioned stimulus (CS) (85 dB). Stimuli presentation was controlled via a personal computer running MED-PC software.

Testing for cued fear conditioning took place in altered chambers (23.5 × 22 × 25.3 cm), housed in sound-attenuating chambers located in a different room. Briefly, altered chambers were constructed with Plexiglas for all four walls and ceiling and a white plastic floor, and the dimensions of the box were different compared to the training context. Additionally, vanilla extract was used as an olfactory cue to further distinguish the altered context chambers from the original training chambers.

Behavioral Procedures

Freezing, defined as lack of movement except for respiration, was measured for the dependent measure. Freezing behaviors were assessed using a time-sampling method where every 10 seconds mice

were observed for 1 second and scored as either freezing or active. Mice were trained and tested in fear conditioning with the protocol described in detail elsewhere.²⁶ Timepoints used for statistical analysis are parenthetically marked where appropriate. Briefly, mice were assessed for their baseline freezing during the first 120 seconds of a 5-minute training session (baseline). Afterward, a coterminating CS (30 seconds, 85 dB white noise)–unconditioned stimulus (US) (2 seconds, 0.57 mA foot shock) pairing was presented. After the cotermination of both the white noise and foot shock, freezing behavior was assessed for 120 seconds following the termination of the first CS–US pairing, and then a second CS–US pairing was administered. The session ended after an additional 30 seconds.

Twenty-four hours after training, mice were placed back in the training chamber and freezing to the context was assessed more than 5 minutes (context). Cued fear conditioning was evaluated approximately 1 hour later in the altered chambers, described previously. Sessions lasted 6 minutes, with the first 180 seconds used to evaluate generalized freezing to the novel context in the absence of a cue (pre-CS) and the second 180 seconds freezing was used to assess freezing to the CS (CS).

Statistical Analysis

Statistical analysis was conducted using the commercially available software package SPSS 18.0. Separate 2 (chronic drug: saline versus nicotine) $\times$ 2 (acute drug: saline versus nicotine) between-subjects analyses of variance were conducted on freezing scores for each age group and separated by the acute dose of nicotine for each timepoint outlined in the procedures (ie, baseline, context, pre-CS, CS). Significant interactions were followed up with simple main effects analysis, examining the effects of the acute drug separately within each chronic drug condition.

Results

To test the hypothesis that sensitivity to nicotine is altered after chronic adolescent nicotine treatment (12.6 mg/kg/day), a lower (0.045 mg/kg), medium (0.18 mg/kg), and higher dose (0.36 mg/kg) of nicotine were administered acutely during the time of training and testing during fear conditioning. Results are presented by the acute dose received during training and testing of contextual fear.

Acute Nicotine: 0.045 mg/kg

Adolescent mice treated with chronic nicotine showed deficits in adult contextual fear that were not reversed with the lower dose of nicotine. Additionally, this dose of nicotine did not enhance contextual learning in adult mice that received chronic saline during adolescence. There was a significant main effect of pretreatment condition when examining freezing to the context ($F(1,23) = 23.04, p < .05$). Specifically, adult mice pretreated with chronic nicotine during adolescence displayed lower freezing scores to the context compared to saline-pretreated mice, regardless of acute drug treatment. This indicates that chronic nicotine administration during adolescence leads to impaired contextual fear learning in adulthood that was not reversed by the 0.045 mg/kg dose of acute nicotine. Additionally, there were no effects on cued learning between groups, and all mice displayed similar levels of cued learning indicating that the lowered freezing to the context seen in the nicotine-pretreated mice was not due to changes in locomotion or motivation. Finally, there were no differences in freezing scores during baseline or the pre-CS timepoints. Further, because the 0.045 mg/kg acute treatment dose did

not enhance contextual fear learning in saline-pretreated mice nor reverse the deficits in nicotine-pretreated mice, no adult comparison group was run for this dose. Group sizes were the following: WCS-Saline, $n = 6$; WCS-Nicotine, $n = 6$; WCN-Saline, $n = 8$; WCN-Nicotine, $n = 7$.

Acute Nicotine: 0.18 mg/kg

There were main effects on contextual fear conditioning for both chronic drug treatment ($F(1,52) = 34.78, p < .001$) and acute drug treatment ($F(1,52) = 12.16, p < .001$). When examining the main effect of pretreatment condition, it was found that adult mice treated with chronic nicotine during adolescence had lower freezing scores to the context than adult mice treated with saline during adolescence. When examining the main effect of acute drug treatment, it was found mice administered acute nicotine had higher freezing scores to the context compared to saline-challenged mice (Figure 1B). However, planned t tests indicated that there was a significant difference between the WCS-Saline and WCS-Nicotine groups, ($t(26) = 3.24, p < .01$), but not between the WCN-Saline and WCN-Nicotine groups ($p > .05$). As with the lower dose, there were no differences in baseline or pre-CS freezing and no differences in cued learning between groups. Thus, a dose of nicotine that routinely causes enhancement of hippocampal-dependent learning did not enhance contextual fear conditioning in adult mice exposed to nicotine during adolescence.^{7,31} Group sizes were the following: WCS-Saline, $n = 14$; WCS-Nicotine, $n = 14$; WCN-Saline, $n = 14$; WCN-Nicotine, $n = 14$.

Adult mice showed enhanced contextual fear with acute administration of nicotine (0.18 mg/kg) regardless of prior chronic drug exposure during adulthood. Specifically, there was a main effect of acute nicotine administration at the 0.18 mg/kg acute dose ($F(1,42) = 21.07, p < .001$). Mice administered acute nicotine had higher freezing to the context than mice treated with saline regardless of pretreatment condition (Figure 2A). While there was a main effect of acute treatment for pre-CS freezing ($F(1,41) = 10.00, p < .05$), with nicotine-treated animals having higher freezing scores than saline-treated animals, there was no difference between groups in freezing to the auditory CS ($p > .05$), indicating nicotine did not have an effect on hippocampus-independent learning. Additionally, because there were no significant differences in baseline freezing, it is unlikely the higher freezing in the context previously associated with a shock in adult mice given acute nicotine was due to increased locomotor activity. Group sizes were the following: WCS-Saline, $n = 10$; WCS-Nicotine, $n = 11$; WCN-Saline, $n = 13$; WCN-Nicotine, $n = 12$. One mouse was in the WCS-Saline group was excluded from the cued test analysis due to escaping the cued box during testing.

Acute Nicotine: 0.36 mg/kg

There was a significant interaction ($F(1,28) = 14.34, p < .001$) and follow-up simple main effects analysis indicate that adult mice pretreated with chronic nicotine during adolescence showed a deficit in contextual fear conditioning and these deficits were reversed with administration of 0.36 mg/kg acute nicotine (Figure 1C). Additionally, mice pretreated with chronic saline during adolescence had no differences in freezing levels to the context when given acute nicotine or saline in adulthood. There were main effects of acute condition during assessments of baseline freezing ($F(1,28) = 17.51, p < .001$), and pre-CS freezing ($F(1,28) = 16.08, p < .001$); however, it is unlikely that increased baseline freezing following acute nicotine solely contributed to enhanced contextual learning in adult

Figure 1. (A–C) Freezing scores of adult animals during varying acute doses of nicotine following chronic nicotine treatment in adolescence. (A) Adult mice that received nicotine in adolescence had lower contextual freezing scores compared to adult mice receiving saline during adolescence. (B) Adult mice that received nicotine treatment during adolescence show lower freezing scores compared to saline controls and acute nicotine (0.18 mg/kg) enhanced contextual learning only in adult mice that received saline in adolescence. (C) Acute nicotine reversed adult deficits in contextual fear resulting from adolescent nicotine treatment. Acute nicotine did not enhance contextual freezing in adult mice treated with saline during adolescence. a^* = main effect of chronic drug treatment, b^* = main effect of acute treatment, $p < .05$, WCS = withdrawal from chronic saline, WCN = withdrawal from chronic nicotine.

Figure 2. (A, B) Freezing scores of adult animals during varying acute doses of nicotine following chronic nicotine treatment during adulthood. (A) There was a main effect of acute nicotine (0.18 mg/kg) administration enhancing contextual fear learning regardless of previous chronic drug administration during adulthood. (B) Acute nicotine administration (0.36 mg/kg) enhanced contextual fear in adult mice receiving chronic saline and chronic nicotine during adulthood. b^* = main effect of acute drug treatment, $p < .05$, WCS = withdrawal from chronic saline, WCN = withdrawal from chronic nicotine.

mice pretreated with nicotine during adolescence as the magnitude of change for contextual fear conditioning was greater than the baseline change. Due to the differences in baseline freezing, the analysis was redone and results were consistent when baseline freezing was

subtracted from contextual freezing scores. There was a main effect of acute treatment on cued learning, whereby mice receiving acute nicotine during the CS presentation had higher freezing scores to the cue previously associated with the shock than mice receiving acute

saline ($F(1,28) = 4.59, p < .05$). However, there were no individual group differences. These results indicate that while the acute dose 0.36 mg/kg increased freezing to the cue there were no significant differences resulting from drug administration in adolescence. Taken together, chronic nicotine pretreatment during adolescence leads to deficits in contextual fear that were only reversed with the highest dose of nicotine. Group sizes were the following: WCS-Saline, $n = 8$; WCS-Nicotine, $n = 8$; WCN-Saline, $n = 8$; WCN-Nicotine, $n = 8$.

In mice beginning chronic drug treatment as adults and given acute nicotine administration (0.36 mg/kg) as adults, there was a main effect of acute drug condition ($F(1,28) = 12.18, p < .01$), whereby mice given acute nicotine during training and testing had higher freezing scores to the context than mice given acute saline regardless of pretreatment condition (Figure 2B). There were significant differences in baseline freezing based on acute drug condition with mice receiving acute nicotine having higher freezing than mice receiving acute saline ($F(1,28) = 5.61, p < .05$), but no significant differences were observed during freezing to the pre-CS or freezing to the cue. WCS-Saline, $n = 8$; WCS-Nicotine, $n = 8$; WCN-Saline, $n = 8$; WCN-Nicotine, $n = 8$.

Conclusions

The major finding from the current experiments is that chronic nicotine exposure during adolescence leads to decreased sensitivity to the effects of acute nicotine on adult contextual fear conditioning. Previous work has demonstrated that acute nicotine given to naive adult mice will enhance contextual fear learning through high-affinity nicotinic receptors in the hippocampus.³⁰ Adult mice that had received chronic nicotine during adolescence required a higher dose of nicotine to enhance adult contextual fear conditioning compared to mice that received chronic saline. In contrast, chronic nicotine exposure during adulthood did not affect responding to acute nicotine in contextual fear conditioning following the same protracted abstinence. Thus, adolescent nicotine exposure not only creates learning deficits in adulthood but also requires a higher dose of nicotine to reverse these learning deficits, which may suggest altered hippocampal cholinergic function.

In adult mice, withdrawal from chronic nicotine administration leads to deficits in contextual fear. These learning deficits in contextual fear persist for up to 4 days and parallel the time course for nAChR upregulation in the dorsal hippocampus.²⁸ Previous work has shown that mice withdrawn from chronic nicotine were more sensitive to the cognitive enhancing effects of acute administration of nicotine compared to mice withdrawn from chronic saline when tested within 2 days of removal of nicotine.²⁵ Adult mice withdrawn from chronic nicotine and administered acute nicotine during training and testing of contextual fear learning had higher levels of freezing compared to mice receiving chronic saline. In the current study, adult mice administered chronic nicotine followed by a protracted abstinence period of 30 days did not demonstrate increased sensitivity to the cognitive enhancing effects of acute nicotine. This is in agreement with previous research that showed nAChR upregulation following chronic nicotine exposure lasts for a discreet period.²⁸ However, it should be noted that adult mice treated with chronic saline as adolescents did not show enhancement with the 0.36 mg/kg dose of acute nicotine, whereas adults treated with chronic saline as adults did. This suggests adolescents may be more sensitive to stress associated with procedures as others have shown.^{32,33} Taken together, this indicates that in adult mice nAChRs are upregulated

during chronic nicotine administration and the receptors are hypersensitive to subsequent nicotine during withdrawal but following a protracted period of abstinence behavioral responding to acute nicotine returns to control levels.

In adolescent mice, chronic nicotine produced long-term learning deficits in contextual fear when tested in adulthood.⁷ Results from the current study suggest that chronic nicotine administration during adolescence leads to decreased sensitivity to the cognitive enhancing effects of acute nicotine administration during contextual fear learning in adulthood. These behavioral findings contrast the results obtained in the adult cohort where prior nicotine treatment had no effect on later responses to acute nicotine. One possible explanation for the persistent deficits in contextual fear and the decreased sensitivity to acute nicotine in adulthood following chronic administration of nicotine in adolescence is altered acetylcholinergic function. Trauth et al.³⁴ found the chronic nicotine administration in adolescent rats caused a persistent reduction in choline acetyltransferase activity, the enzyme responsible for the synthesis of acetylcholine, in the midbrain as well as decreases in HC-3 binding in the hippocampus.³⁴ HC-3 binding is specific to the high-affinity presynaptic choline transporter and reductions correspond to decreased cholinergic signaling.³⁴ Thus, nicotine exposure during adolescence may reduce hippocampal cholinergic function leading to decreased sensitivity to the effects of acute nicotine on learning. These findings offer one possible explanation for why younger initiation of nicotine use can lead to more severe dependence in adulthood as larger amounts of nicotine may be consumed in an attempt to compensate for cognitive deficits and decreased cholinergic function.

A limitation from the current study is increased freezing following acute nicotine during baseline and pre-CS measures, which could contribute to the observed reversal of the learning deficit in the adolescent cohort. It should be noted that the 0.36 mg/kg dose only reversed the learning deficit so that freezing levels were comparable to the WCS-Saline group and did not enhance contextual learning compared to WCS-Saline group, which further supports the hypothesis that chronic nicotine exposure during adolescence attenuated the cognitive enhancing properties of acute nicotine. Previous work from our group has determined that increased generalized freezing following acute nicotine treatment, measured during exposure to an altered context during the pre-CS time period, does not preclude the interpretation that acute nicotine enhanced contextual fear learning.^{26,35} Finally, a possible ceiling effect in testing for the cued-shock association could explain the lack of a treatment effect in the CS test. Previous work demonstrated acute nicotine had no effects in cued conditioning when the training parameters changed from two CS-US pairings to a single CS-US pairing and when the cue presentation was reduced from 30 to 15 seconds, suggesting that the effects of acute nicotine are specific to hippocampus-dependent learning and not due to a ceiling effect during the CS test.³⁶ Further, chronic and acute administration of nicotine does not change shock sensitivity in adult rodents.^{37,38} Although similar fear conditioning parameters were used in the current study compared to the previously published work, it is still unknown if chronic nicotine during adolescence would impact cue-induced freezing if there were a single CS-US pairing or if the shock levels were lowered. However, given the consistent findings that acute nicotine enhances while chronic nicotine disrupts contextual learning with no effects in cued learning in both adolescent and adult models of nicotine exposure, it is likely that the effects seen in the current study do not reflect a ceiling effect.

In summary, results from this study suggest that interventions to reduce or abstain from nicotine use should be specifically tailored to address differences between individuals with adolescent or adult onset of nicotine use. Current tobacco cessation treatments point to a combination of nicotine replacement therapies (ie, transdermal patches, lozenges, varenicline, bupropion, etc.) in conjunction with behavioral therapies focused on identifying and addressing cravings and other cues that may lead to relapse.³⁹ Only recently have cognitive impairments during nicotine withdrawal been implicated in relapse and lowered success in long-term abstinence.^{6,40,41} Results from the current study indicate cognitive impairments may persist following nicotine exposure during adolescence and that attempts to self-medicate with nicotine during adulthood may require higher doses; this could perpetuate nicotine addiction. Thus, emphasizing ways to cope with cognitive impairments alongside the traditional pairing of nicotine replacement therapies and behavioral therapies could improve long-term nicotine abstinence in individuals who initiated nicotine use in adolescence. In addition, adolescent nicotine exposure leads to both short-term and long-term cognitive deficits with earlier age of initiation leading to more deficits later in life.^{6–9} Since cognitive impairments are often reported as a reason for maintaining tobacco use,⁴¹ nicotine use during adolescent could promote continued use by creating learning impairments that only higher doses of nicotine can reverse.

Funding

This work was funded with grant support from the National Institute of Drug Abuse (DA017949 to TJG) and by the Weinstein Summer Graduate Award presented by the Roslyn and Stephen Weinstein of the Civic Foundation.

Declaration of Interests

None declared.

Acknowledgments

The authors would like to thank Sheree Logue and Prescott Leach for their contributions to the experimental design and implementation.

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