THE EFFECTS OF ADOLESCENT NICOTINE EXPOSURE ON ADULT MEMORY FOR STIMULUS ATTRIBUTES AND EXTINCTION LEARNING

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By

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Introduction

Tobacco consumption is a growing problem in the United States, killing over 400,000 people a year (Centers for Disease Control and Prevention [CDC], 2008). Moreover, adolescent nicotine use is on the rise, with nearly 4,000 American children under the age of 18 beginning to smoke each day (CDC, 2010). Given that development of the mature nervous system continues into late adolescence (Slotkin, 2002), it is important to investigate and understand the effects of adolescent nicotine exposure on the fully developed adult nervous system. More specifically, research is needed that investigates how adolescent nicotine exposure affects adult behavior, learning, and memory.

Animal models of nicotine exposure have proven to be a valuable tool in investigating the psychological and physiological effects of this drug. Research on the effects of nicotine exposure on learning and memory has, until recently, typically focused on adult exposure. These studies traditionally find that nicotine enhances learning and memory in adult animals. For instance, nicotine was found to enhance spatial learning and memory (Levin, Kaplan, & Boardman, 1997; Liu, Wang, Sun, & Wang, 2004; Poincheval-Fuhrman & Sara 1993) and trace fear conditioning (Davis & Gould, 2007; Gould, Feiro, & Moore, 2004; Raybuck & Gould, 2010), as well as reverse age-related memory impairment in rats (Arendash, Sanberg, & Sengstock, 1995).
Exposure to nicotine in adult animals also results in enhanced synaptic plasticity in the hippocampus (Placzek, Zhang, & Dani, 2009) and in cortico-limbic circuits (Mansvelder, Mertz, and Role, 2009). Behavioral evidence of this enhanced synaptic communication has been provided by Thomas Gould’s lab; Gould found evidence that nicotine exposure in adult animals enhances context learning, but not context-shock associative learning (Gould & Whener, 1999; Kenney & Gould, 2008). Specifically, it was discovered that nicotine administered prior to acquisition of contextual information in the context pre-exposure facilitation effect paradigm enhanced contextual learning, a hippocampal-dependent task. However, nicotine did not appear to enhance learning or retrieval of fear conditioned to the context, which relies on both hippocampal and amygdala processing (Kenny & Gould, 2008). Since contextual information surrounding a training event is thought to rely on hippocampal processing, and nicotine has been found to enhance hippocampal plasticity through the upregulation of nicotinic acetylcholine receptors (nAChRs) (Placzek, et al., 2009), Gould’s finding is not surprising. However, nicotine’s enhancement of synaptic plasticity and the resulting improvement in learning and memory seems to disappear if nicotine exposure occurs during adolescence (Fountain, Rowan, Kelley, Wiley, & Nolley, 2008; Slotkin, 2002; Vaglenova, Birru, Pandiella, & Breese, 2004; Xu, Seidler, Cousins, Slikker, & Slotkin, 2002).

There is increasing evidence that nicotine acts as a teratogen, an agent that disrupts normal physiological development, when administered during pre- or post-natal development. A recent study discovered that nicotine, if administered prenatally, resulted in hyperactivity and retarded growth in post-natal animals; increased levels of anxiety
behaviors, as evidenced by impaired performance on an elevated plus maze, in adolescent animals; and cognitive impairments, as evidenced by impaired active avoidance learning, in adult animals (Vaglenova, et al., 2004). This study also examined the effects that nicotine exposure and withdrawal have on rearing. The authors used a cross-fostering procedure in which nicotine-exposed offspring were reared by control (saline exposure) mothers and control offspring were reared by nicotine exposed mothers. Valgenova et al. (2004) found that both nicotine-exposed offspring and control offspring reared by nicotine-exposed mothers showed high levels of anxiety behavior in the elevated plus maze. More recently, an investigation of adolescent nicotine exposure on adult cognition revealed cognitive impairments resulting from exposure to the teratogen in a serial pattern learning procedure (Fountain, et al., 2008). Specifically, Fountain et al. (2008) discovered that injections of nicotine 5 days per week for 5 weeks during adolescence impaired adult rats’ ability to learn serial patterns as nicotine-exposed rats made fewer correct responses than controls. Taken together, these data suggest that nicotine exposure during early development produces long-lasting behavioral and developmental deficits.

Similar changes in anxiety behaviors have been discovered to result from adolescent exposure to nicotine, as well. Adolescence appears to represent a critical period in the development of the nervous system that remains highly vulnerable to damaging agents, such as drugs, radiation, and infections (van Gelder, van Rooij, Miller, Zielhuis, de Jong-van den Berg, & Roeleveld, 2010). In a recent experiment, rats were exposed to daily nicotine injections during adolescence (postnatal days 30-44) and tested
for anxiety and depressive behaviors in adulthood (Iniguez, et al., 2009). The authors discovered that when tested in adulthood (one month following nicotine cessation), animals that received adolescent nicotine exposure exhibited shorter latencies to floating and spent more time floating in a forced swim task than control animals (saline exposure), which is typically interpreted as evidence/presence of depressive behaviors. Adolescent nicotine exposure also resulted in an increase in anxiety-like behaviors when animals were tested in adulthood on the elevated plus maze (Iniguez, et al., 2009). Rats that received nicotine injections during adolescence spent significantly less time in the open arms of the maze, had a lower percentage of arm entries, and spent significantly more time grooming in the closed arms than control animals, which is interpreted as higher anxiety levels among the nicotine animals. These data are further support that adolescence represents a critical period for normal biobehavioral development and that nicotine exposure during this period impairs that development. However, it remains unclear how long these changes in behavior will persist. The cited studies tested the animals at a relatively young age (postnatal days 45-55). The transition into adulthood in rats is not clearly defined, and it is possible that animals this age are still making the transition from late adolescence into adulthood. Further, these studies did not investigate the time course of these behavioral deficits. To gain a better understanding of the severity and longevity of nicotine’s deleterious effects, animals need to be tested at a much later age.

In addition to affecting anxiety and depressive behaviors, adolescent nicotine exposure has also been found to damage the developing nervous system. Findings from
studies on the physiological effects of adolescent nicotine exposure typically point towards nicotine’s damaging effects on developing brain cells (Slotkin, 2002). For instance, administration of nicotine during adolescence alters the expression and function of serotonin receptors and thus impairs synaptic functioning in the hippocampus and cerebral cortex (Xu, Seidler, Cousins, Slikker, & Slotkin, 2002), which may underlie the depressive symptoms observed in those animals. Nicotine has also been found to alter cell differentiation to affect synaptic maturation, and it evokes permanent changes in synaptic activity and cell signaling (Slotkin, 2002). The importance of these findings is further highlighted by the discovery that brain development continues into late adolescence (Slotkin, 2002).

Recent work with animal models of adolescent nicotine exposure has uncovered further evidence of neural damage. Specifically, adolescent nicotine exposure resulted in a decrease in total cell number in the cerebral cortex, midbrain, and hippocampus (Slotkin, 2002). It was also discovered that hippocampal precursor cells prematurely undergo apoptosis (programmed cell death) when exposed to nicotine (Oliveira-da-Silva, et al., 2009; Xu, et al., 2003). This cell damage is not as prevalent as the damage occurring from fetal exposure, but as Slotkin (2002) points out in his review paper, the number of cells in the brain does not typically change once neural mitosis is completed. In other words, most neural cells that are damaged during adolescent nicotine exposure are not repaired or regenerated, which implies that any damage done to the developing brain during adolescence is likely to be permanent.
A recent study provided some of the first behavioral evidence of the long-term hippocampal damage due to adolescent nicotine exposure. Andrea Spaeth and her colleagues (2010) investigated the effects of adolescent nicotine exposure on context conditioning, a hippocampal-dependent task, in adult rats. In this study, male and female rats were exposed to nicotine exposure via an osmotic minipump from postnatal days (PD) 28-42 (i.e., adolescence) at a dose of either 0, 3.0, or 6.0 mg/kg/day of nicotine bitartrate. These animals were then trained and tested in a context conditioning/lick-suppression paradigm as adults, or PD 65-70. Water deprived animals were placed in a conditioning chamber and trained to drink from water-filled lick tubes. Context conditioning involved placing the animals into the conditioning chamber in the absence of the water tube and administering either 10 unsignaled footshocks or no shocks. The animal then presumably associates the context, consisting of visual, olfactory, and auditory stimuli, with the footshock and learns to fear that context. It has been well documented that the hippocampus is necessary for the encoding of contextual information and that disruption of this brain structure is associated with deficits in contextual learning/memory (Kim & Fanselow, 1992; Maren, Aharonov, & Fanselow, 1997; Marschner, Kalisch, Vervliet, Vansteenevink, & Buchel, 2008). The authors therefore hypothesized that if adolescent nicotine damages developing hippocampal cells and results in an impaired hippocampus, animals that received adolescent nicotine should exhibit impaired contextual conditioning.

Spaeth and her colleagues (2010) found evidence supporting this hypothesis. They tested the animals for conditioned fear in the same context in which they received
shocks. Fear was measured as latency to lick the water tubes. Higher lick suppression (subsequently more freezing) is indicative of fear, and therefore suggests successful contextual fear conditioning. Animals exposed to adolescent nicotine, at either dose, showed significantly lower latency-to-lick than the saline treated animals. However, the adolescent nicotine-exposed animals still had higher latency scores than did animals that received no shock, suggesting that some context conditioning, and therefore hippocampal processing, occurred. This behavioral finding supports the physiological findings that exposure to nicotine during adolescence impairs normal hippocampal development.

Spaeth et al. (2010) also investigated the effects of adolescent nicotine exposure on delayed conditioning to a discrete cue, a learning paradigm that does not rely on the hippocampus, but on amygdala processing (Ledoux, 2000; Spaeth, et al., 2010). Animals were given 10 tone-shock pairings to train fear to a tone in the training context. Animals were tested for fear to the tone in a context distinctly different from training. Exposure to adolescent nicotine did not significantly impair delayed conditioning. In other words, all animals exhibited comparable levels of fear to the tone and all had similar latencies to lick the water tube in the presence of the tone. Further, adolescent nicotine exposure also had no effect on extinction learning. Rats received extinction trials, presented with the conditioned stimulus in the absence of the unconditioned stimulus, after testing for context conditioning. After animals were tested for context conditioning, all animals were exposed to the context in the absence of the footshock. Adolescent nicotine exposure failed to impair extinction learning in adult rats.
Spaeth et al. (2010) is among the first investigations into the long-term behavioral effects of adolescent nicotine exposure on a hippocampal-dependent learning task. This study was able to provide evidence that the hippocampal damage resulting from adolescent nicotine results in learning and memory impairments that rely on this structure. However, there are several short-falls in this study. First, the animals received nicotine exposure from PD 28-42. This is defined loosely as the adolescent period by Spear (2000). However, due to normal variability among animals, some may have either entered adolescence slightly before this period or may have exited adolescence after this period. To be sure that all animals received nicotine exposure during the entire adolescent period, nicotine administration should occur slightly before and after this adolescent window. Another issue not addressed by this study is the question of the longevity of nicotine’s effects. Spaeth et al. (2010) only tested animals in early adulthood. It remains unclear for how long this hippocampal-dependent learning impairment will persist. Spaeth’s study also does not delineate context learning from context-shock learning. In Kenney and Gould’s (2008) study, they discovered that adult nicotine exposure enhances context learning, while having little or no effect on context-shock learning. Since it is evident that nicotine differentially affects the two types of learning in adulthood, it is reasonable to assume to that adolescent nicotine may also affect the two in different ways. The current study attempts to extend the findings from Spaeth et al. (2010) and remedy the highlighted issues.

The current study employs a passive-avoidance paradigm that Riccio and his colleagues have extensively utilized (Richardson & Riccio, 1983). In the passive-
avoidance model, Riccio has been able to study both the animal’s central memory for the
event, such as the response required and the outcome, and the memory for the peripheral
aspects of the event, such as the context in which the training took place (Briggs &
Riccio, 2007; Richardson & Riccio, 1983). In this learning paradigm, animals are trained
in a distinct context to fear the dark compartment of a black/white shuttle box. Animals
are then tested for fear of the dark compartment in a similar, but different context. Riccio
and his colleagues consistently find that the animal’s memory for the contextual stimuli
decays over time, while the fear memory remains intact, resulting in the animal’s
generalizing its fear from the training context to novel contexts. In other words, if tested
immediately, the animals do not show fear to the black side of the shuttle box in a new
context (i.e. they exhibit the ‘context shift effect’). However, after a period of time, the
memory for contextual cues decays, and the animals come to fear the black side in the
new context. Therefore, this paradigm allows for the assessment of fear learning separate
from contextual learning and is ideally suited to investigate the effects of adolescent
nicotine on a hippocampus-dependent task.

Experiment 1 uses the passive-avoidance paradigm to investigate the effects of
adolescent nicotine exposure on adult memory for contextual attributes. Since the
encoding of contextual information relies heavily on the hippocampus, any deficit in
hippocampal processing resulting from nicotine exposure will be expressed as an increase
in the rate of the forgetting of contextual cues, i.e. greater fear generalization between the
two contexts. We therefore hypothesize that animals exposed to nicotine during
adolescence will show increased fear responding and a deficit in a hippocampal-
dependent memory task. Specifically, we expect to find adolescent nicotine exposure resulting in an increase in the forgetting of the contextual cues surrounding the training event, as evidenced by a transfer of conditioned fear from the training context to the testing context. Further, this study attempts to address the short-falls experienced in Spaeth et al.’s (2010) study by increasing the exposure period to slightly before and after Spear’s defined adolescent period and by training and testing the animals well into adulthood. Given the physiological evidence of nicotine-induced damage discussed in the prior sections, we hypothesize that adolescent nicotine exposure will result in long-lasting, relatively permanent impairments in hippocampal-dependent learning and memory.

We were also interested in expanding on Spaeth et al.’s finding that adolescent nicotine exposure had no effect on extinction learning. We wanted to know whether a longer extinction exposure as well as administering the extinction treatment and testing at a much later time would result in the same null findings. Therefore, in Experiment 2, animals were placed back into the feared context for an extinction trial and were re-tested for fear in the original context. Since extinction learning typically does not rely heavily on the hippocampus and Spaeth’s (2010) study found no significant effect of nicotine on extinction, we hypothesized that adolescent nicotine exposure would have little to no effect on extinction learning in adult animals.
Experiment 1

Experiment 1 was designed to investigate the effects of adolescent nicotine exposure on the forgetting of contextual cues in adult rats. To accomplish this, a passive avoidance procedure was used to assess the animal’s memory for both the central event, in this case conditioned fear to the black side of a black/white shuttle box, and the peripheral attributes, such as the contextual information surrounding the fear stimulus. Both males and females were used in this experiment to determine if an interaction between nicotine exposure and sex exist in this learning task. Several studies have found evidence that adolescent nicotine may affect males and females differently. For instance, one study discovered that damage to hippocampal cells, both neurons and astrocytes, resulting in impaired synaptic functioning was more profound in female rats than in males (Xu, et al., 2003). Behavioral studies investigating sex differences in vulnerability to adolescent nicotine exposure on animal cognition have recently discovered sex-selective effects of drug exposure. Stephen Fountains’s lab recently discovered that that nicotine appeared to retard learning in male rats more than in female rats when trained and tested in a serial pattern learning paradigm (Kolar, et al., 2010; Meduri, Pickens, Rowan, Bevins, & Fountain, 2010). Therefore, Experiment 1 investigates the effects of adolescent nicotine exposure on adult memory for contextual cues separately for males and females to determine if the drug differentially affects rates of forgetting of the contextual cues surrounding the fear conditioning.
method

Subjects

119 (58 female and 61 male) hooded Long Evans rats from a breeding colony in the Department of Psychology at Kent State University (180-190 days of age) were individually housed with free access to food and water in a room maintained on a 15/9 light/dark cycle. To increase generality and to examine potential sex differences, we used both male and female rats in each experiment. All procedures were conducted in a facility accredited by the Association for Assessment and Accreditation and Laboratory Animal Care. Kent State University’s Institutional Animal Care and Use Committee approved all protocols in this paper.

Nicotine Exposure

At post-natal day 21, rats were weaned, sexed, and segregated into boxes of three (same dose group). Adolescent nicotine injections began at post natal day 25 in which rats received daily intraperitoneal (i.p.) injections at specified dose for 35 days. Post natal day 25 is considered to be the adolescent development period for rats as they are developed enough to be weaned but not old enough to be considered adults, which is around post natal day 60 (Spear, 2000). We use PD 60 as a conservative estimate of adulthood in rats. There is no absolute age at which rats enter into and out of adolescence. Spear (2000) loosely defines the adolescent period as the age range in which most animals are expected to “exhibit adolescent-typical neurobehavioral characteristics”
which she states occurs between PD 28 and PD 42. Therefore, we chose to expose animals to nicotine slightly before and well after this defined adolescent period to ensure that animals received exposure throughout the entire adolescent period. Animals were randomly assigned to one of five dose groups: 0mg/kg (1.0 mg/kg saline), 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, and 1.0 mg/kg of nicotine. Rats received their last injection on post natal day 59 and began a no drug/break period. This drug free period was included in the design to ensure that all animals were not going through withdrawal from nicotine cessation at the time of testing. Animals typically show altered behavior (Hamilton, Berger, Perry, & Grunberg, 2009) as well as a decrease in cognitive ability, such as learning and memory, when experiencing withdrawal from most drugs (Andre, Gulick, Portugal, & Gould, 2008; Davis & Gould, 2009; Davis, James, Siegel, & Gould, 2005; Portugal & Gould, 2009). We wanted to be sure that any data collection was not confounded with withdrawal periods. During post natal days 59-148, the rats were used for a serial pattern learning procedure in the Fountain laboratory. At post natal day 180, rats started context shift training.

**Apparatus and Contexts**

Training and testing occurred in two identical 43.18 X 17.78 X 17.78 cm shuttle-boxes with grid floors. A guillotine door divides each shuttle-box into two equal-sized compartments – one black and one white. The two shuttle-boxes are located in different rooms that serve as contexts. Context A is a 1.6 X 2.33 meter room brightly lit with house fluorescent lights containing white walls and posters serving as visual cues. Context B is a 1.83 X 2.74 m room dimly lit by a 25-w red light bulb containing white walls, white
noise (76db), and is scented with Glade PlugIns Scented Oil Apple Cinnamon air freshener.

_**Training**_

All animals were handled for five minutes on two consecutive days prior to the beginning of the experiment. This handling procedure serves to acclimate the subjects to the presence of the experimenter. This is especially important in a fear paradigm experiment, because fear of the experimenter would potentially confound the experiment. Initially, the rats were given a brief 30 second exposure to the context. This brief exposure to the context serves to introduce the animal to the training context and allows the animal to encode the contextual cues. Each rat was then placed in the white compartment of the shuttle-box facing away from the closed guillotine door. After 20 seconds, the door was raised and the latency to cross into the black compartment was measured (in seconds). The door was then lowered and two inescapable foot shocks (0.75 mA, lasting 1 sec) were administered at 5 sec and 10 sec. Ten seconds after the second footshock, animals were removed and returned to their home cage.

_**Testing**_

Testing consists of one 10-minute passive-avoidance session identical to the training trial, except that no shocks were administered and the guillotine door remained open. During testing, the rat was placed on the white side and allowed to choose between the white and black compartments. The total time spent on the safe (white) side (TTW), as measured in seconds, was recorded as dependent measures (fear of the black compartment). Higher TTW is associated with more fear generalization and more
forgetting of contextual cues (in the Shift context). We use TTW scores as a measure of fear and learning due to the fact that an untrained animal prefers and will spend most of the 10 minute trial in the black compartment. Therefore, by resisting the instinct to cross into the black side and spending most of time in the white side, we consider the animal to be exhibiting fear of the black side. Total time on white is also used as our primary measure of fear over latency to cross into the black compartment as it is considered to be a more representative measure of fear memory (Santucci & Cardiello, 2004). Santucci and Cardiello (2004) argue that the initial cross into the black compartment during testing serves as a sufficient reminder of the training period and activates the neural circuitry involved in fear memory retrieval. Therefore, an animal that was previously trained to fear the black compartment may cross into the compartment early in the testing phase, but due to the reactivation of that fear memory, remain in the white compartment for the duration of the test. For this reason, TTW scores are thought to be a more reliable measure of fear to the black compartment than latency to cross.

Procedure

Training in the passive avoidance task began at postnatal day 180 (with a range of post natal days 180-190). All animals received fear training in Context A in which they are conditioned to fear the dark side of the shuttle box, as described above. Testing occurred 5 days later. Each nicotine dose condition (0mg/kg, 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, and 1.0 mg/kg) was divided into two context groups. Half of the animals received training in Context A and testing in Context B (Shift groups). The other half of the animals were tested in the same context they received training in (Same groups). We used
a stratified random sample procedure to randomly assign animals within each dose group to either the Same or Shift conditions. The Same groups serve as the controls for context training and provide benchmarks for comparison. The 0mg/kg nicotine group also serves as a control for nicotine and will be used to determine what effects nicotine has on behavior. As described above, cross-through latency and total time on white were measured, and the effects of adolescent nicotine exposure on the forgetting of contextual cues were assessed. It should be noted that we expected to find very little generalization in the control group (0mg/kg), as 5 days is not enough time to facilitate natural forgetting. We did expect some forgetting of contextual cues to occur, but not enough to inhibit our ability to assess nicotine’s effects on forgetting. Five days was chosen because we thought that it would provide a more sensitive test for even the lowest dose of nicotine. Presumably, testing only 24 hours later would not be as sensitive due to the strength of the memory. At five days, the memory for contextual cues is more vulnerable to manipulations as it has already begun to weaken. Also, it is important to note that ideally a control group with a short retention interval (an immediate test – 0Day) would have been included in the study to serve as another control/comparison group. However, including such a group would have reduced the already low sample size of approximately six animals per group per dose. For this reason, the group was not added.
Results and Discussion

This experiment utilized several analyses of variance (ANOVA) to examine the effects of adolescent nicotine exposure on adult memory for stimulus attributes. Certain assumptions that the ANOVA makes about the data must be checked in order to determine whether this analysis is the best method for the data. A Kolmogorov-Smirnov test (K-S test) was performed to determine the data’s deviation from normality (Massey, Jr, 1951; Rosenthal, 1968). The K-S test indicated that TTW scores statistically significantly deviated from a normal distribution, $D(119) = 0.25$, $p<.01$. For the reason that data transformations failed to normalize the distribution of data and the widely accepted belief that the ANOVA is relatively robust to violations of normality (Glass, Peckham, & Sanders, 1972), we chose to run the analyses on the untransformed data. A Levene’s test of Equality of Error Variances was conducted on TTW scores and indicated that the variances are statistically significantly different, $F(19, 99) = 3.24$, $P<.001$, indicating the homogeneity of variance assumption was violated. However, the ANOVA is quite robust to violations of homogeneity of variance assumptions when sample sizes are equal (Rogan & Keselman, 1977). Therefore, it seems unlikely that the degree of bias resulting from the violation of the homogeneity of variance assumption will inhibit our ability to use the ANOVA as a means of data analysis.

During training, initial cross-through latency to cross into the black compartment from the white compartment was recorded. This latency is a measure of initial fear to the
passive avoidance chamber, of which there should be none. The latency score is also used to check for non-specific effects that nicotine may have on mobility. An analysis of variance (ANOVA) was performed on initial cross-through latencies and revealed no statistically significant differences between groups, \( F(9,109) = 1.15, p>.05 \). This indicates that all animals had similar responses to the apparatus at the beginning of the study regardless of nicotine exposure. The lack of differences in cross-through latencies also indicates that nicotine exposure does not result in mobility deficits. This is an important point because it rules out the possibility that higher TTW scores during testing are due to non-specific effects of drug treatment on mobility. Therefore any group(s) exhibiting higher TTW scores during testing are more likely exhibiting fear to the black compartment rather than remaining immobile due nicotine exposure.

Figure 1 shows grand mean (+ SEM) TTW scores for males in all dose and context groups. Male rats in the Shift0mg/kg group had lower TTW scores (lower avoidance) than male rats in the Same 0mg/kg group. This difference indicates the context shift effect is present at 5 days following fear training. Exposure to injections of nicotine during adolescence impaired memory for stimulus attributes at all doses. Figure 2 shows grand mean (+ SEM) TTW scores for females in all dose and context groups. Female rats in the Shift0mg/kg group did not differ from female rats in the Same0mg/kg on TTW scores, indicating that there was no context shift effect present at 5 days.

A three way ANOVA on TTW scores yielded no main effect of Drug Dose, \( F(4,99) = 1.25, p>.20, \eta_p^2 = .05 \), indicating there was no statistically significant difference
between the nicotine doses. There was a main effect of Context (Shift vs. Same), $F(1, 99) = 12.94$, $p<.01$, $\eta^2_p = .12$, indicating that there was a difference between contexts (i.e. higher TTW scores for nicotine control animals in the Same context than in the Shift context). This difference defines the boundaries of the context shift effect at 5 days. There was also a main effect of Sex, $F(1, 99) = 4.47$, $p<.05$, $\eta^2_p = .04$, indicating a difference between males and females (higher overall TTW scores for females). The Dose by Context interaction was found to be non-significant, $F(4, 99) = 1.95$, $p>.10$, $\eta^2_p = .07$. The Dose by Sex interaction was found to be non-significant, $F(4, 99) = 0.96$, $p>.40$, $\eta^2_p = .04$. The Context by Sex interaction was also found to be non-significant, $F(1, 99) = 2.08$, $p>.15$, $\eta^2_p = .02$. However, there was a significant interaction between Dose, Context, and Sex, $F(4, 99) = 2.72$, $p<.05$, $\eta^2_p = .09$.

A simple effects test was performed to determine the nature of the statistically significant interaction. As figures 1 and 2 indicate, the only visual difference between groups appeared to be within the males between the nicotine control Shift and nicotine control Same groups (i.e. context shift effect). Simple effects test indicated that the male nicotine control Shift group (0mg/kg_Same) differed significantly from the male nicotine control Same group (0mg/kg_Shift), $F(1, 99) = 22.54$, $p<.001$. This statistically significant difference between groups defines the boundaries of the context shift effect at 5 days. Specifically, this difference indicates a statistically significant forgetting of stimulus attributes in the 0mg/kg_Shift group. We were also interested in the effects of adolescent nicotine exposure on the adults’ memory for stimulus attributes. Therefore, a Simple effects test was performed to determine the relationship between the male
nicotine control Shift group and the different nicotine dose groups within the Shift condition. The simple effects test indicated that males exposed to nicotine at all doses (0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, and 1.0 mg/kg) had statistically significantly higher TTW scores in the Shift condition compared to males that received no nicotine (0 mg/kg), \( F(4, 99) = 5.33, p<.01 \).

These data indicate that exposure to adolescent nicotine impaired memory for stimulus attributes in a non-dose-dependent fashion for adult male rats. This is evidenced by the finding that males across all nicotine doses had higher TTW scores than the 0mk/kg_Shif group. Further, nicotine animals in the Shift groups had comparable TTW scores to animals in all dose groups in the Same conditions, indicating that the contexts were treated similarly among nicotine animals. However, no differences were found between female controls and female nicotine groups. This may be due to the fact that the context shift was not present at 5 days in female controls. More specifically, female controls exhibited near maximum TTW scores in both Context groups. The lack of difference between Shift and Same nicotine controls (0mg/kg) indicates that females in the Shift condition generalized their fear of the apparatus to the new context after 5 days. For this reason, any differences between nicotine animals and non-nicotine animals could not be assessed. Future studies need to employ a shorter retention interval between training and testing to ensure female controls show the context shift effect in order to better assess adolescent nicotine exposure’s effect on the forgetting of contextual cues.
Experiment 2

Experiment 1 provided evidence that adolescent nicotine exposure results in an increase in the forgetting of contextual cues in adult male rats. More specifically, we found that fear trained in Context A transferred at a higher rate in males that received nicotine exposure during adolescence. Next we wanted to know whether the extinction of that fear memory would be affected by nicotine exposure. Therefore, Experiment 2 examined the effects of adolescent nicotine on adult extinction learning. Animals from Experiment 1 were given an extinction trial in the original training context. All animals were tested for fear of the black side of the shuttle box immediately following extinction in the same context (A).
METHOD

Subjects
119 male and female Long-evans rats (180-190 days old). This experiment used the same animals from Experiment 1.

Nicotine Exposure
All animals received nicotine or saline injections as described in Experiment 1. Adolescent nicotine injections began at post natal day 25 in which rats received daily i.p. injections at specified dose for 35 days. Animals were randomly assigned to one of five dose groups: 0mg/kg (1.0 mg/kg saline), 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, and 1.0 mg/kg of nicotine. Rats received their last injection on post natal day 59 and began a no drug/break period.

Training
All animals had already received fear training as described in Experiment 1. Animals received two footshocks (.75 mA) in the black side of the shuttle-box.

Extinction
All rats received an extinction trial in the original training context (Context A). Animals were placed in the black (feared) side of the apparatus for 6 minutes and were not permitted to escape. Animals received no shocks during this period. After the 6 minute extinction trial, animals were returned to the home colony for 15 minutes.

Testing
Fifteen minutes following return to the home colony, rats were then tested for fear of the black side using the same 10 minute test procedure described in Experiment 1. The TTW scores were recorded as the dependent measure.
Results and Discussion

The data were checked to ensure that they met the assumptions required by the ANOVA. A K-S test indicated that TTW scores following extinction training statistically significantly deviated from a normal distribution, \( D (119) = 0.23, p<.01 \). We chose to run the analyses on the untransformed data as transformation did not improve normality. Levene’s test of Equality of Error Variances indicated that the variances are statistically significantly different, \( F (19, 99) = 5.09, P<.001 \). However, due to the fact that this experiment has equal sample sizes (6 per group) we believe it robust to violations of homogeneity of variance and that our use of the ANOVA as a means of data analysis was not overly biased by the violation of the homogeneity of variance assumption.

Figure 3 shows grand mean (± SEM) TTW scores for males in all dose and context groups following extinction training. The Context was kept as a variable in the analysis of extinction learning to determine if the context that animals were tested in from Experiment 1 (either the same or shifted context) had any effect on their ability to extinguish fear to the apparatus. Little extinction was observed across conditions in male rats as evidenced by relatively high TTW scores. Though a slight resistance to extinction appears to result from adolescent nicotine exposure compared to nicotine control animals, this difference is not very large. Exposure to injections of nicotine during adolescence does appear to impair extinction learning in adult male rats. Figure 4 shows grand mean (± SEM) TTW scores for females in all dose and context groups following extinction.
Very little extinction of fear was observed in female rats as evidenced by high TTW scores. This failure to extinguish appeared independent of adolescent nicotine exposure as well as the context in which these animals were tested for fear in Experiment 1. These results suggest that adolescent nicotine exposure does not affect extinction learning in adult rats.

A three way ANOVA on TTW scores yielded no main effect of Drug Dose, $F(4, 99) = 1.73, p>.10, \eta^2_p = .06$, indicating comparable levels of extinction across all dose groups (0mg/kg, 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, and 1.0 mg/kg of nicotine. There was no main effect of Context (Shift vs. Same), $F(1, 99) = 3.56, p>.05, \eta^2_p = .04$, indicating the context in which animals received testing in had no effect on extinction of fear in the training context. There was a main effect of Sex, $F(1, 99) = 4.06, p<.05, \eta^2_p = .04$, indicating a difference in extinction learning between males and females. Specifically, males had an overall greater amount of extinction, as evidenced by lower TTW scores following extinction training, than females. The Dose by Context interaction was found to be non-significant, $F(4, 99) = 0.48, p>.70, \eta^2_p = .02$. The Dose by Sex interaction was found to be non-significant, $F(4, 99) = 0.83, p>.50, \eta^2_p = .03$. The Context by Sex interaction was also found to be non-significant, $F(1, 99) = .17, p>.60, \eta^2_p = .01$. Finally, the interaction between Dose, Context, and Sex was to be non-significant, $F(4, 99) = 1.14, p>.30, \eta^2_p = .04$.

These data indicate that exposure to adolescent nicotine did not significantly effect extinction learning in adult male and female rats. It is possible that we failed to
find an effect of nicotine exposure on extinction learning due to the short extinction trial the animals received. Six minutes may not have been a long enough exposure to the feared stimulus to produce a significant reduction in fear. This in turn may have obscured our ability to detect any effects the nicotine may have had on extinction learning. Longer extinction exposures should be employed by future studies to further investigate adolescent nicotine exposure on adult extinction learning. We did find evidence that male rats were able to extinguish more fear than the female rats during the 6 minute extinction session.
General Discussion

The current study investigated the effects of adolescent nicotine exposure on the forgetting of contextual cues surrounding a fear conditioning trial in adult rats. Male rats exposed to 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, and 1.0 mg/kg of nicotine during adolescence (PD25-59) showed impaired memory for contextual cues, a form of memory dependent on the hippocampus, in a non dose-dependent fashion compared to that of the control animals (1.0mg/kg Saline). All doses of nicotine resulted in male rats with higher TTW scores that were comparable to the TTW scores exhibited by animals in the Same conditions, indicating greater levels of fear generalization between the two contexts. However, these data only partially support the stated hypothesis that adolescent nicotine exposure results in an increase in the forgetting of the contextual cues surrounding the training event as no differences were found between female controls and female nicotine groups. Further, the current study found no significant effects of adolescent nicotine exposure on extinction learning in adult rats. Animals in both context groups in all doses (1.0 mg/kg Saline, 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, and 1.0 mg/kg nicotine) exhibited similar levels of extinction learning.

The data presented in the current study partially support Spaeth et al.’s (2010) finding that animals exposed to adolescent nicotine exhibit impaired context conditioning when tested in adulthood, thereby providing further behavioral evidence that this adolescent exposure impairs hippocampal functioning. Spaeth et al. (2010) discovered
that adolescent nicotine exposure impairs contextual fear conditioning when tested in adulthood (PD 65-70). The current study provided similar evidence of hippocampal impairment as it was discovered that memory for contextual cues was impaired at 5 days in adult male rats. The current study was able to extend on Spaeth et al.’s (2010) findings and provide some of the first evidence that hippocampal impairment resulting from adolescent nicotine appears to be relatively stable and permanent. Adult male rats that received adolescent nicotine exposure exhibited impaired contextual memory when tested at PD170. The Spaeth study tested animals at PD 65-70, which is considered early adulthood. The current study provides evidence that the memory impairment for contextual cues persists well into late adulthood without reversing or mitigating.

Speath et al. (2010) administered either 3.0 or 6.0 mg/kg of nicotine during adolescence, which is claimed to induce plasma levels found in moderate to heavy smokers. However, it is unlikely that adolescent users of nicotine begin their habits with moderate to heavy use. Therefore, the current study employed much lower doses in an attempt to not only mimic the lower use of adolescent smokers, but also to determine how sensitive the developing nervous system is to a teratogen such as nicotine. Animals were injected with 0.03, 0.1, 0.3, or 1.0 mg/kg nicotine and it was expected that only the higher doses would produce contextual memory deficits. However, adult male rats at all nicotine doses exhibited similar impairments for memory for stimulus attributes. This indicates that the developing nervous system, in particular the hippocampus appears vulnerable to even small doses of the teratogen nicotine. One explanation for this surprising vulnerability is that even though small doses of nicotine were administered, animals
received chronic exposure to the drug (35 days of injections). Further, the nicotine injections were given slightly before the accepted onset of adolescence and continued well after adolescence is said to end. This prolonged nicotine exposure that occurred throughout the entire adolescent period may be responsible for the longevity of contextual memory impairment. In other words, the developing nervous system may be vulnerable to even small doses of nicotine if the exposure persists throughout development, ending well into adulthood.

The current study only partially supports Spaeth et al.’s (2010) findings that all animals exposed to adolescent nicotine show impaired memory for contextual information in adulthood due to a lack of control effect in female rats. No differences were found between female rats that received adolescent nicotine exposure and female control rats. This lack of difference between the nicotine groups and control is due to the fact that female control (Saline) rats in the Shift condition failed to show a context shift effect. More specifically, female controls exhibited near maximum TTW scores in both Context groups. The lack of difference between Shift and Same nicotine controls (0mg/kg) indicates that females in the Shift condition generalized their fear of the apparatus to the new context after 5 days. For this reason, any differences between nicotine animals and non-nicotine animals could not be assessed. This finding may indicate that female rats naturally forget contextual cues at a faster rate than male rats. Future studies need to employ a shorter retention interval between training and testing to ensure female controls show the context shift effect in order to better assess adolescent nicotine exposure’s effect on the forgetting of contextual cues. In addition, an
investigation into the natural rates of the forgetting of contextual cues for males and females may be warranted to determine if differential forgetting rats exist between the two sexes. This could potentially prove valuable if there are in fact different rates of generalization between males and females as it would demonstrate the need for using both sexes in experimental designs investigating generalization.

It is also possible that the failure to find a context shift effect in female controls may be due to small sample size (6 animals per group per sex) and had a large number of comparisons made between the three variables (Drug – with five doses, Sex- male and female, and Context – Same and Shift). For this reason, we may have not adequate statistical power to detect any subtle differences that may exist between the female controls and the female rats that received adolescent nicotine exposure. However, given the low variability and standard error of the means for all female context and dose conditions, it is unlikely that low power or small sample sizes resulted in the lack of a context shift effect in female rats. It is more likely that the lack of context shift effect resulted from a difference in the natural rates of forgetting of contextual cues between the two sexes as discussed above.

Although Experiment 2 confirmed the hypothesis and Spaeth’s (2010) finding that adolescent nicotine exposure does not appear to impair extinction learning in adult animals, a design limitation exists that may limit the generalizability of the current study’s findings. Since all animals in the second experiment were the same animals that were used in the first experiment, not all animals started the extinction trial with the same
levels of fear. Specifically, some of the animals, even in the nicotine conditions, crossed over to the black side during the 10 minute testing session. Though these rats may have only spent a brief amount of time on the black side of the shuttle box, they were exposed to the conditioned fear stimulus in the absence of the unconditioned stimulus (the shock), which is exactly what an extinction trial is. Therefore, some of the animals received more extinction than other animals that never crossed into the black side during the test session. This prior experience with no shock in the black side for some of the subjects may have affected the total time on white scores in the second experiment, thereby limiting the conclusions that can be drawn from this study. However, this is not perceived as a major problem in the current study due to the finding that there were no statistically significant differences between nicotine animals and controls in either of the context conditions (Shift or Same). Future investigations into the effects of adolescent nicotine exposure on adult extinction learning in which all animals have identical extinction exposures are required to better understand nicotine’s influence on this type of learning.

The data from the current study suffer from an interpretive problem that must be acknowledged and discussed. It remains unclear what higher TTW scores actually indicate in the current paradigm. With the context shift paradigm the assumption is made that the increased rates of fear generalization between the two contexts that result from adolescent nicotine exposure are due to an increase in the forgetting of contextual cues. However, we may actually be measuring a failure to learn the contextual cues instead of measuring forgetting. The nicotine may alter the animals’ ability to learn and encode these cues. The current study design is not capable of distinguishing between the two and
cannot determine if the higher TTW scores exhibited by male rats that received adolescent nicotine exposure resulted from a failure to learn or a failure to remember the contextual cues surrounding the training event. Future investigations that utilize the same paradigm should include the immediate retention interval that was omitted from the current study due to an inadequate sample size. An immediate test would help determine if the animals learned the contextual information and could recall it while the information was still in the short-term memory phase. If the animals that received adolescent nicotine exposure show memory for contextual cues during the immediate test, the increased generalization rates at later retention intervals can be said to result from a memory impairment rather than a learning impairment. Regardless of whether adolescent nicotine results in impairment in either learning contextual information or remembering it, the current study highlights the long-term deleterious effects of adolescent nicotine exposure on the developing nervous system.

An important question that the findings from the current study generate is whether this adolescent nicotine-induced memory impairment of contextual cues can be reversed. The data point to a relatively long-term impairment of a hippocampal-dependent task and support previous research indicating that exposure to the teratogen during a critical period of nervous system development results in severe and in some cases relatively permanent damage (Adriani, et al., 2004; Oliveira-da-Silva, Manhaes, Cristina-Rodrigues, Filgueras, & Abreu-Villaca, 2010; Oliveira-da-Silva, et al., 2009; Slotkin, 2002; Xu, et al., 2002). However, as was discussed earlier in this paper, nicotine exposure during adulthood appears to upregulate the cholinergic system and results in an improvement in learning...
and memory (Davis & Gould, 2007; Gould, Feiro, & Moore, 2004; Levin, Kaplan, & Boardman, 1997; Liu, Wang, Sun, & Wang, 2004; Poincheval-Fuhrman & Sara 1993; Raybuck & Gould, 2010). One important issue that needs to be addressed in future research is whether re-exposure to nicotine in adult animals can reverse the impairments in hippocampal functioning, and related learning and memory performance, that result from adolescent nicotine exposure.

The present findings indicate that exposure to adolescent nicotine results in a long-term impairment of a hippocampal-dependent task in adult male rats. Male rats exhibited greater rates of fear generalization between two contexts than control animals. Prior research has indicated that exposure to adolescent nicotine results in damage to developing brain cells (Slotkin, 2002) and impairs synaptic functioning in the hippocampus and cerebral cortex (Xu, et al., 2002). The data from the current study provide some of the first behavioral evidence suggesting that adolescent nicotine exposure results in long-term impairment of contextual memory, indicating a relatively stable and permanent impairment of hippocampal functioning. Future studies investigating adolescent nicotine exposure’s effects on memory for contextual information should be aimed at further investigating the teratogen’s influence in adult female rats by decreasing the retention interval in an attempt to attain the context shift effect in control animals. A context-shift effect at a 24 hour retention test should result in a context shift in female control rats and allow for a comparison of forgetting rates between control and nicotine animals. Further, the reversibility of this hippocampal/memory impairment should be investigated by re-administering nicotine in adulthood in an
attempt to discover any possible mitigating effects adult nicotine exposure may on the damaged system.
Figure 1. Grand mean (± SEM) total time spent on the safe (TTW) side in seconds for male groups in Experiment 1. Groups labeled as “Shift” were tested in the shifted context (B). Same and Shift groups at 0 mg/kg represent the context shift effect. The Shift groups at 0.03, 0.1, 0.3, and 1.0 mg/kg nicotine demonstrate a statistically significant generalization of fear to the novel context (B).
Figure 2. Grand mean (+ SEM) total time spent on the safe (TTW) side in seconds for female groups in Experiment 1. Groups labeled as “Shift” were tested in the shifted context (B). Same and Shift groups at 0 mg/kg represent the absence of the context shift effect. No differences in the forgetting of contextual cues were discovered between animals exposed to adolescent nicotine (0.03, 0.1, 0.3, and 1.0 mg/kg nicotine) and control (0 mg/kg nicotine).
Figure 3. Grand mean (± SEM) total time spent on the safe (TTW) side in seconds for male groups following extinction training in Experiment 2. All animals received extinction exposure and were tested in Context A. Groups labeled as “Shift” were initially tested in the shifted context (B) in Experiment 1. No differences in extinction learning were discovered between animals exposed to adolescent nicotine (0.03, 0.1, 0.3, and 1.0 mg/kg nicotine) and control (0 mg/kg nicotine) in either context condition (Shift or Same).
Figure 4. Grand mean (± SEM) total time spent on the safe (TTW) side in seconds for female groups following extinction training in Experiment 2. All animals received extinction exposure and were tested in Context A. Groups labeled as “Shift” were initially tested in the shifted context (B) in Experiment 1. No differences in extinction learning were discovered between animals exposed to adolescent nicotine (0.03, 0.1, 0.3, and 1.0 mg/kg nicotine) and control (0 mg/kg nicotine) in either context condition (Shift or Same).
References


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