Adolescence as a Unique Period of Vulnerability to Environmental Stressors: Age-Dependent and Sex-Dependent effects of Nicotine Exposure on Anxiety Utilizing The Light-Enhanced Startle Paradigm

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Adolescence as a Unique Period of Vulnerability to Environmental Stressors: Age-Dependent and Sex-Dependent effects of Nicotine Exposure on Anxiety Utilizing The Light-Enhanced Startle Paradigm

A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in the Interdisciplinary Studies Neuroscience Program from The College of William & Mary

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Adolescence as a Unique Period of Vulnerability to Environmental Stressors:
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Abstract

This study utilized a rodent model of anxiety in which the defensive behavior of rats provides a measure of operational anxiety known as “Light-Enhanced Startle” (LES; Walker & Davis, 1997). The focus of the study was to explore age-dependent and sex-dependent vulnerabilities to the anxiety-producing effects of nicotine in rats. An additional goal was to establish the LES paradigm for use in adolescent animals. The effect of acute first-time exposure to nicotine on the magnitude of light-enhanced startle in adolescent versus adult rats was measured. Adolescent and adult animals did not have similar dose-response patterns revealing age-dependent differences in nicotine’s effect on anxiety. Additionally, these different dose-response patterns depended on sex. In general, nicotine was anxiogenic in most conditions and females showed stronger anxiogenic responses to nicotine than males. Collectively, outcomes reveal age-dependent vulnerabilities to the anxiety-producing effects of nicotine that depend on drug dose and sex, and establish the light-enhanced startle paradigm for use as an animal model of anxiety expression in adolescence, which is viewed as a critical developmental period. Adolescence as a unique period of vulnerability to the effects of stress and advantages of the LES paradigm as an experimental model of anxiety are also discussed.
Adolescence as a Unique Period of Vulnerability to Environmental Stressors:
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Nicotine use is highly comorbid with psychiatric disorders including depression (Iñiguez et. al., 2009) and schizophrenia (Ziedonis et al., 2008), and in animal models has been implicated in learning and memory impairments (Spaeth et al., 2010). Additionally, there is a strong link between anxiety and smoking. Smoking rates are much higher among individuals diagnosed with General Anxiety Disorder (46%) and Panic Disorder (35.9%) compared to those without a psychiatric disorder (22.5%; Ziedonis et al., 2008). Individuals with an anxiety disorder also display faster progression from initial use of nicotine to nicotine dependence (Kushnar et al., 2012). Further, 82 percent of adult smokers report trying their first cigarette before the age of 18 and adolescents with anxiety and depression symptoms are twice as likely to report nicotine dependence (Centers for Disease Control, 1994; McKenzie et al., 2010). These latter observations underscore adolescence as an especially vulnerable period not only for the potential of nicotine exposure to produce dependence, but also for nicotine’s impact on anxiety. Nicotine, the major psychoactive ingredient in tobacco, has been demonstrated to increase stress and anxiety in human users, despite tobacco users claiming anxiolytic and stress reducing properties as a major basis for their tobacco use (Abreu-Villaca, 2003; Parrott, 1999). For example, Newhouse et al. (1992) found that nicotine-naïve humans exposed to acute nicotine reported increases in anxiety and tension rather than relaxation. Relatedly, Parrott (1999) proposed that the pattern of human tobacco use and the development of nicotine dependency actually causes stress, which can then be further exacerbated by nicotine withdrawal symptoms. Other authors have found no evidence to support the hypothesis that smoking has a role in causing increases in stress over time, and suggest instead that stress is an etiological factor contributing to smoking (Wills, Sandy, & Yaeger, 2002). Thus the relationship between smoking and anxiety, including anxiety disorders, is nuanced. It may be the case that
tobacco use potentiates the development of these disorders. On the other hand, these disorders might predispose individuals to nicotine use, possibly manifested as an individual's attempt to “self-medicate” with tobacco products to relieve their symptoms.

The present research investigates the effect of first-time exposure to nicotine on anxiety in adolescents and adults in an animal model of anxiety known as Light-Enhanced Startle (LES). A brief overview and critique of current models of anxiety is presented below and is followed by discussion and predictions for the present experiment.

**Overview of Anxiety Paradigms**

Common methods for measuring anxiety behavior in animals include Fear-Potentiated Startle, the Elevated Plus Maze, and the Light-Dark Box. Perhaps the most well-developed paradigm encountered in anxiety literature is Fear-Potentiated Startle (FPS), developed by Brown et al. (1951). In FPS, a conditioned cue (such as a tone) is followed by an aversive footshock in order to establish a learned Pavlovian association between the tone and shock. During the test, the conditioned cue (i.e., tone) is presented in the absence of shock and is followed by presentation of a brief but loud burst of white noise (the acoustic startle pulse). The acoustic startle pulse elicits an unlearned whole-body “flinch” reaction called the acoustic startle reflex. The magnitude of the acoustic startle reflex can be quantified by transducer platforms upon which the rat sits and measures displacement in terms of grams of force. Fear-potentiated startle is observed when the magnitude of startle reactions to the noise is larger on tone+noise test trials than on noise-alone test trials. This enhancement of the acoustic startle response is taken to reflect a greater level of fear and anxiety elicited by the shock-associated tone (Brown et al., 1951).

Another commonly encountered animal model in the anxiety research literature is the Elevated Plus maze (EPM) illustrated in Figure 1 (image source: http://www.mpipsykl.mpg.de/en/institute/services/emolab/index.html).
The EPM paradigm consists of four elevated arms, two of which have high walls (known as the “closed” arms), and the other two arms are without walls (known as the “open” arms; e.g., Walf & Frye 2007). Since rats and mice are at most risk of predation in open, exposed areas, they will tend to avoid these open areas, modeled by the open arms of the EPM. Therefore, it is supposed that animals spending more time in the open arms are less anxious than animals spending less time in the open arms.

The Light-Dark Box paradigm is illustrated in Figure 2 (image source: http://www.mpipsykl.mpg.de/en/institute/services/emolab/index.html).

Animals are placed in the box which has two separate chambers, one that is brightly lit, and one that is dark, and allowed to explore freely. Operationally, animals that spend relatively more time in
the light chamber are less anxious than those that spend relatively less time in the light chamber, since rats have an unlearned aversion to brightly lit spaces (Chaouloff, Durand, and Mormède, 1986). The underlying cause for the anxiogenic effect of bright open spaces in rats and mice is most likely due to their nocturnal nature. In evolutionary terms, when in settings of bright light, such as in the daylight, rats and mice may be at greater risk of predation (Walker & Davis, 1997).

Critique of Animal Anxiety Paradigms

The Light-Enhanced Startle paradigm used in the present research was developed in part to overcome limitations of the above described animal models of anxiety as used in behavioral and pharmacological neuroscience (Walker & Davis, 1997). One major issue with the Fear-Potentiated Startle paradigm is that it requires a learned conditioned association to be established before anxiety can be assessed. As described above, this paradigm measures anxiety elicited by an associatively conditioned cue (e.g., tone previously paired with shock). Therefore, a drug’s effect on behavior indicative of anxiety in this paradigm may be difficult to interpret: it may be a result of the drug’s effect on anxiety per se, or the drug could affect memory retrieval of the learned association (Walker & Davis, 1997). Furthermore, Davis et al. (2010) more recently characterized fear measured with FPS (which he termed “phasic” fear) as anatomically distinct from other forms of fear that might be better characterized as anxiety (which he called “sustained” fear) as measured by the remaining paradigms described above. Specifically, evidence suggests phasic fear is mediated by projections of the medial division of the central amygdala, whereas anxiety (sustained fear) is mediated by the activation of the lateral divisions of the central amygdala. Lateral divisions of the central amygdala release corticotropin releasing hormone, causing longer term effects in the bed nucleus of the stria terminalis, a brain system associated with anxiety, which is operationally measured by the light-enhanced startle paradigm in this report (Davis et al., 2010).

A conceptual distinction may be made among the common and prevailing anxiety paradigms described above. Fear-potentiated startle involves a reflex-based response measure in
which the acoustic startle response that is reflexively elicited by a startle pulse or burst of white noise. In contrast, the EPM and Light-Dark Box paradigms require a “voluntary” act by the organism to enter arms or spaces of the maze, presumably mediated by a balance of drives for exploratory behavior and avoidance of unsheltered open areas. Drugs that affect behavior in these latter paradigms could be doing so by affecting cognitive mechanisms responsible for choice, rather than anxiety per se (see Picciotto, Brunzell, & Caldarone, 2002).

**The Light-Enhanced Startle Paradigm**

In 1997 Walker & Davis introduced what would become known as the Light-Enhanced Startle (LES) paradigm. In LES, animals are placed in a chamber in a movement restricting cage on top of a platform that can measure downward force applied to it. Presentation of acoustic startle pulses (usually 100-ms presentations of 90-115 dB bursts of white noise) elicits the acoustic startle reflex as described earlier. The critical manipulation in LES is the illumination of the testing chamber: startle-eliciting pulses are presented either in the dark, or in the presence of a bright light. Because bright light is an innately aversive stimulus to rodents and presumed to be associated with anxiety, startle responses to noise stimuli in the bright light condition are expected to be enhanced compared to startle responses to the same noise stimuli in the dark condition. Moreover, relative levels of startle magnitude in the light condition can be assessed after administration of psychopharmacological agents to evaluate the agent's effect on anxiety (Walker & Davis, 1997).
Walker & Davis (1997) demonstrated that startle amplitude to noise pulses is enhanced in a brightly lit chamber compared to a dark chamber, an effect that persisted after repeated testing. This is the light-enhanced startle effect. Presumably, anxiety produced by the bright light enhances reactivity to the startle-eliciting noise stimuli. An important distinction between LES and FPS paradigms, both of which involve a startle enhancement during states of fear, is that startle enhancement associated with LES is not based on prior associative learning. Therefore, the effects of drugs on startle in LES cannot be attributed to the effect of the drug on learning or memory retrieval. Additionally, because LES measures changes in reflexively elicited rather than voluntarily produced behavior, variation in LES performance cannot be attributed to the effects of experimental manipulations including drugs on cognitive mechanisms of choice. The LES paradigm has been validated as an animal model of anxiety: known anxiolytics such as buspirone, chlordiazepoxide, and flesinoxan decrease the magnitude of LES (Walker & Davis, 1997, 2002; de Jongh et al., 2002). Moreover, some pharmacological anxiogensics such as yohimbine have been demonstrated to increase the magnitude of FPS but not LES, establishing pharmacological differentiation.
between FPS and LES (see also Bijlsma et al., 2010). In sum, LES has become a validated animal model of anxiety that overcomes some of the limitations of earlier models. Its use has further provided evidence for pharmacological and anatomical dissociation of different forms of fear.

A phenomenon analogous to LES has also been established in humans, known as the Dark-Enhanced Startle effect. Humans, who are diurnal animals and thus more evolutionarily vulnerable to predation in the dark, will display elevated startle reactions in darkness compared to light (Davis et al., 2010).

Despite advantages of the LES paradigm as a model of anxiety there are very few reports examining how anxiety is affected by nicotine in this paradigm, and no reports comparing anxiogenic (or anxiolytic) effects of nicotine in young or adolescent versus adult animals. In one paper utilizing non-adult rats in LES, Weber et al. (2003) examined the age at which LES and FPS first emerges. Because LES does not require formation of learned associations whereas FPS does, Weber et al. (2003) predicted that LES would emerge earlier than FPS. They found that high illumination enhanced startle amplitudes and this enhancement emerged at postnatal day 18 but FPS did not emerge until postnatal day 23. These findings are consistent with the idea that the neural mechanisms involved in LES are distinct from those involved in FPS (Weber et al., 2003; see also Hunt et al., 1997).

**Adolescence, Nicotine, and Anxiety**

Adolescence has long been characterized as a period of unique development including significant changes in social and risk-taking behavior as well as cognition due, at least in part, to ontogenetic changes in the brain (Spear, 2000). Adolescents display greater amounts of reckless and impulsive behavior and are less sensitive to the negative effects of drug intake including reduced expression of withdrawal, which may contribute to heightened risk of dependence (Trimpop, Kerr, & Kickcaldy, 1999). Differential effects of nicotine in adolescents and adults have been reported. Adriani et al. (2003) observed that periadolescent rats self-administer nicotine at
higher levels than postadolescent counterparts, thus implicating adolescence as critical period of
development characterized by increased vulnerability to neurobehavioral effects and addictive
properties of nicotine. Kupferschmidt et al. (2010) investigated the effects of acute nicotine
exposure (0.40, 0.80 mg/kg) on anxiety in the EPM and light-dark box. Adolescents expressed
higher anxiety in general in the EPM (had fewer open arm entries and less time spent in the open
arms compared to adults) and nicotine actually reduced this effect, partly attributable to nicotine’s
sensitizing effect on locomotion. Nicotine did not differentially affect adolescent versus adult
performance in the light-dark box (see also Schochet, Kelley, & Landry, 2004; Cruz, DeLucia, &
Planeta, 2005). However, Slawecki et al. (2003) examined the long-term consequences of
adolescent nicotine exposure on anxiety in the open field paradigm (conceptually similar to the
light-dark box paradigm), and found that five consecutive days of transdermal nicotine exposure in
adolescence increased anxiety in adulthood relative to controls. In sum, there is evidence for
differential effects of nicotine in adolescents compared to adults as well as evidence (albeit mixed)
supporting nicotine’s anxiogenic profile.

Anxiety & Gender

There is evidence that stress may affect males and females differently and that adolescence
may be a particularly sensitive period for the expression of such sex differences. For example,
Weintraub et al. (2010) examined gender differences in response to the environmental stressor of
social isolation (rats housed individually) during adolescence (postnatal days 30-50). They
observed that, after adolescent social isolation and relative to group-housed controls, female rats
had an enhanced release of the stress hormone corticosterone in response to acute and repeated
restraint stress compared to males. In another study, Elliott et al. (2004) exposed male and female,
adolescent and adult rats to nicotine (saline, 0.10, 0.50, or 1.00 mg/kg) for several days and then
tested using the EPM. Adolescent females exhibited increased anxiety (decreased percent time
spent in open arms of EPM) whereas males did not. Adult males and females did not differ in their
response to nicotine, which was anxiogenic. This research extends the earlier indication of nicotine’s anxiogenic profile and further indicates that sex-dependent effects may emerge, especially in adolescence.

**Goals and Predictions**

In one of the few reports examining nicotine’s effects on anxiety as measured by LES, Jonkman et al. (2008) found that nicotine withdrawal increased startle amplitude in LES suggesting LES can be used to model anxiety-associated withdrawal symptoms. These authors examined the effect of nicotine withdrawal during abstinence (i.e., following nicotine exposure), not the direct effect of nicotine on anxiety. Moreover, at present there are no published studies examining LES in adolescents. Given known relationship between nicotine and stress, and unique vulnerabilities of adolescence discussed earlier, the present research sought to more directly assess nicotine’s impact on anxiety measured in LES in adolescent and adult rats. Male and female, and adolescent and adult, rats were exposed to acute nicotine followed immediately by LES testing. It was predicted that adolescent rats exposed to nicotine would show a greater enhancement in LES amplitude compared to adults. It was additionally predicted that females would display a greater enhancement in LES in response to acute nicotine than males.

**Method**

**Subjects**

Sixty-nine adolescent (n=34 male, n=35 female) and 48 adult (n=24 male, n=24 female, see Footnote 1) drug naïve albino Sprague-Dawley rats obtained from the breeding colony of The College of William & Mary's Psychology Department were used as subjects. At the beginning of LES testing, adolescent animals were postnatal day (PD) 45 and adult animals were PD 80. Rats, weaned at PD 21, were individually housed in wire mesh cages measuring 25 x 18 x 18 cm. Animals were maintained on a 14:10 hour light-dark cycle and had access to food and water *ad*
libitum. Temperature and humidity were vivarium controlled. Animal care and experimental treatments were in compliance with IACUC.

**Nicotine**

Nicotine hydrogen tartrate salt was dissolved in saline into two dosages: 0.15 mg/ml and 0.40 mg/ml and titrated to a pH of 7.4. Nicotine was injected intraperitoneally in a volume of 1 ml/kg body weight.

**Apparatus**

Light-enhanced startle testing occurred in three identical small startle cages. The startle cages served as functional restraint cages (Coulbourn Instruments, Model E05-15 for adolescents and Model E05-20 for adults) which were themselves placed inside larger sound and light-attenuating startle chambers (Coulbourn Instruments, Model E10-24). The sides and bottom of each startle cage were constructed of flat plastic, and the lid of each cage consisted of a rounded convex steel-bar grate that could be clasped to the frame of the cage. Startle cages measured 18.5 cm x 11 cm x 9.5 cm for adults or 16 cm x 8.5 cm x 8.0 cm for adolescents (height measured from base of plastic floor to top of convex lid). Each startle cage could be placed inside its own larger startle chamber such that animals could be trained and tested individually. The startle chamber consisted of a wooden box lined with sound-attenuating acoustic foam padding and the interior of the chamber measured 52 cm X 52 cm X 30.5 cm (L x W x H). The center of each chamber lid housed a 7.5 cm high-frequency speaker that could deliver a 50-ms burst of white noise (rise time 0 ms) that served as the stimulus to elicit startle reactions. The amplitude of startle stimuli delivered by the speaker was controlled by software and varied between 100 dB, 105 dB and 115 dB (C scale). Each startle cage was placed upon a 1-lb (adolescents) or 5-lb (adults) maximum output transducer platform (Coulbourn Instruments, Model E45-11: 1-lb platform for adolescents and Model E45-15: 5-lb platform for adults) located beneath the speaker of the chamber lid such that the distance
between the speaker and lid of the startle cage was 15 cm. Startle reactions to the 50-ms noise bursts were measured by strain gage load cells which served as response sensors in the transducer platforms. The transducer recorded voltage displacement proportional to the force applied to it and peak voltage displacement occurring within the first 200 ms from startle stimulus onset transformed into grams of force served as the dependent measure.

A 26-W, General Electric compact fluorescent light bulb could be turned on to provide bright illumination of the chamber (1876 lux, measured from the center of the startle platform). The bulb was centered on the left interior wall of the startle chamber and was positioned 19 cm from the chamber floor. In this position the bulb was located 30 cm from the center of the startle cage which held the animal during sessions. All data recording and stimulus delivery was controlled by LabLinc V (Coulbourn Instruments) hardware and software.

**Light-Enhanced Startle Test Procedure**

Each LES test session was comprised of two 20-min phases with each phase separated by a 5-minute rest period. During Phase 1 rats were placed in the startle chamber and after 300 s were exposed to 30, 50-ms startle stimuli, ten at each of three different dB amplitudes (100 dB, 105 dB, 115 dB). The interstimulus interval (ISI) between each startle stimulus was 30 s. The distribution of different dB startle stimuli was pseudo-randomly distributed such that a given dB startle stimulus could occur no more than two times in succession and that the different amplitude startle stimuli occurred in blocks of three (i.e., [115→100→105] → [105→115→100]). Following the completion of Phase 1, rats were removed from the chambers and exposed to a 5-minute rest period in a separate transport cart. At the end of the 5-minute rest period rats were returned to startle chambers and Phase 2 was initiated. Phase 2 was an identical replication of Phase 1. During Phase 2, 30 startle stimuli at each of the three different dB amplitudes were presented during the 20-min session. Phase 1 and Phase 2 were discriminated by the presence or absence of the bright light. During “Dark-Dark” test sessions, both Phase 1 and Phase 2 were conducted in the dark with no
chamber illumination. During “Dark-Light” test sessions, Phase 1 was conducted in the dark and Phase 2 was conducted in the presence of the bright light. A mean startle amplitude score averaging across all Phase 1 trials and a separately computed mean startle amplitude score averaging across all Phase 2 trials was computed for each animal.

Test 1 and Test 2 were preliminary tests in the absence of nicotine. These tests were conducted in order to establish the basic LES effect and to assess the presence of LES in adolescent animals. Test 3 was the critical LES plus nicotine test. During Test 3, the effect of nicotine on the magnitude of LES in adolescent and adult animals was evaluated.

**Test 1 (Dark-Dark).** During Test 1 all animals were exposed to LES testing in the absence of nicotine. Both Phase 1 and Phase 2 were conducted in the dark, in the absence of the bright light. Adolescent animals were PD45 and adults were PD80 on Test 1. Animals remained in home cages with no treatment for two days following Test 1.

**Test 2 (Dark-Light).** During Test 2 all animals were exposed to LES testing in the absence of nicotine. Phase 1 was conducted in the dark and Phase 2 was conducted in the presence of the bright light. Adolescent animals were PD48 and adults were PD83 on Test 2. Animals remained in home cages with no treatment for three days following Test 2.

**Test 3 (Dark-Light, Nicotine).** Prior to Test 3 animals were counterbalanced into drug condition (saline, 0.15, or 0.40 mg/kg nicotine) with respect to weight, litter, and sex, for both adolescents and adults. During Test 3 all animals were exposed to LES testing and the effect of acute nicotine exposure on the magnitude of LES was assessed. Phase 1 was conducted in the dark and Phase 2 was conducted in the presence of the bright light. Test 3 was identical to the procedure of Test 2 except that on Test 3, animals received a single intraperitoneal injection of saline, 0.15 mg/kg or 0.40 mg/kg nicotine at the beginning of the 5-minute rest period that separated Phase 1 and Phase 2. Adolescent animals were PD52 and adults were PD87 on Test 3.
Results and Discussion

Can LES Be Used to Study Anxiety in Adolescent Animals?

Raw peak startle data were analyzed to assess the presence of the basic LES effect on Day 1 (Dark-Dark) and Day 2 (Dark-Light). Preliminary 2 (Day: Dark-Dark vs. Dark-Light) X 2 (Phase: Ph1 vs. Ph2) within-subject ANOVAs were conducted separately in adult and adolescent animals. In adult animals the Day X Phase ANOVA revealed significant main effects of Day, $F(1,47) = 34.83, p < .001$, and Phase, $F(1,47) = 46.42, p < .001$, as well as a Day X Phase interaction $F(1,47) = 32.99, p < .001$. In adolescent animals the Day X Phase ANOVA similarly revealed significant main effects of Day, $F(1,68) = 66.47, p < .001$, and Phase, $F(1,68) = 99.23, p < .001$, and a Day X Phase interaction $F(1,68) = 173.55, p < .001$. As can be observed in Figure 4 which displays data for adult animals, on Day 1 (Dark-Dark), as expected, there was no change in peak startle between the first and second dark phases. On Test Day 2, however, there was an increase in startle amplitude from the dark phase to light phase. This is the light-enhanced startle effect. In Figure 5, displaying data from adolescent animals, a similar pattern emerged. There was no change in peak startle amplitude across phases of Day 1 (Dark-Dark), but there was a significant increase in peak startle amplitude across phases of Day 2 (Dark-Light). These outcomes validate the Light-Enhanced Startle phenomenon as first described by Walker and Davis (1997). Pertinent to the present research, these outcomes establish the Light-enhanced startle phenomenon in adolescent animals.

Percentage change scores were used to evaluate the effect of nicotine on the magnitude of LES on Day 3, the critical nicotine exposure day. Percentage change scores are commonly used to
assess the effects of drugs in startle paradigms and were used in the present report in order to accommodate differences in raw peak startle likely to occur across animals different in size (e.g., adolescent vs. adult; see Walker & Davis 1997). Percentage change scores were computed as 
\[(\text{Phase 2 Peak Startle} – \text{Phase 1 Peak Startle})/\text{Phase 1 Peak Startle}\) X 100. Percentage change scores were computed for each animal and then were collapsed across animals within condition to generate individual group means. ANOVAs and, when appropriate, follow-up comparisons were conducted using analysis-specific error terms \((p = .05)\).

Does the Effect of Nicotine Depend on Age, Sex, or Both?

Percentage change data from the Day 3 nicotine test were analyzed in a 2 (Age: adolescent vs. adult) X 3 (Drug: saline, .15 mg/kg nicotine, .40 mg/kg nicotine) X 2 (Sex: male vs. female) ANOVA. The analysis revealed significant main effects of Drug \(F(2, 105) = 8.67, \ p < .001\), Sex \(F(1, 105) = 8.54, \ p = .004\), a Drug X Sex interaction \(F(2, 105) = 3.04, \ p < .05\), as well as a 3-way Drug X Sex X Age interaction \(F(2, 105) = 3.00, \ p < .05\). The source of the 3-way interaction appears to be the fact that the dose-response curves were generally similar for males and females in adolescents (Figure 6) but different for males and females in adults (Figure 7). This possibility was supported by Drug X Sex interaction contrasts conducted separately for adolescent and adult animals which failed to reveal a Drug X Sex interaction for adolescents, \(F(2, 63) < 1\), but did reveal a marginally significant Drug X Sex interaction for adults, \(F(2, 42) = 3.03, \ p = .059\). For adolescents there was a significant main effect of Sex, \(F(1, 63) = 14.62, \ p < .001\), and females demonstrated significantly higher LES than males in each of the two drug groups, \(Fs(1, 63) > 6.95\), but males and females did not differ in the saline control. In adults, males and females did not differ despite the numerical trend for females to demonstrate higher LES in .40 mg/kg as well as saline conditions.
These outcomes indicate that there are differences between how adolescents and adults respond to acute nicotine with respect to anxiety levels and these differences further depend on sex. As apparent in Figure 6, adolescent females tended to express higher levels of LES than males but the dose-response pattern was generally similar in males and females. However, as seen in Figure 7, adult males and females did not have similar patterns of LES responding across drug dose. Taken together, these outcomes reveal age-dependent variation in acute nicotine’s effect on anxiety. Adolescents and adults do not respond in the same way to acute nicotine.

These data also reveal pronounced sex differences. In adolescents (Figure 6), drug-exposed females had significantly higher levels of light-enhanced startle than males. In adults (Figure 7), females demonstrated a nonsignificant trend toward higher LES than males (except at the .15 mg/kg dose). These results imply that female adolescents are slightly more sensitive to the anxiety-producing effects of nicotine. Conversely, these patterns could be suggested to indicate that adolescent males are more tolerant to the aversive anxiogenic effects of acute nicotine compared to females, a possibility considered in more detail later.

Is Nicotine Anxiogenic?

Nicotine was anxiogenic in both adolescent and adult animals. In adolescents the main effect of Drug was significant, $F(2, 63) = 6.27, p = .003$. As suggested by Figure 6, compared to saline controls, nicotine significantly enhanced LES for males at the .15 mg/kg dose, $F(1, 63) = 4.61$, and for females, nicotine significantly enhanced LES at both .15 mg/kg and .40 mg/kg doses, $F_{s}(1, 63) \geq 4.39$. In adults the main effect of Drug was also significant, $F(2, 42) = 4.09, p = .02$. For adult males, as suggested by Figure 7, nicotine significantly enhanced LES at the .15 mg/kg dose, $F(1, 42) = 6.77$, and for adult females, nicotine significantly enhanced LES at the .40 mg/kg...
dose, $F(1, 42) = 6.53$.

These outcomes indicate that nicotine, when acutely administered to nicotine-naïve animals, is generally anxiogenic. These results are consistent with the literature implicating nicotine as an anxiogenic substance. In no condition was there evidence for nicotine having an anxiolytic effect on operational anxiety.

**Do Adolescents Have a Greater Anxiety Response to Nicotine Than Adults?**

In order to directly compare the effect of nicotine in adult versus adolescent animals, 2 (Age: adolescent vs. adult) X 3 (Drug: saline, .15 mg/kg, .40 mg/kg) ANOVAs were conducted separately on data for males and females. Data from Figures 6 and 7 are replotted in Figures 8 and 9 to illustrate relevant age comparisons. The Drug X Age ANOVAs revealed significant main effects of Drug for both males (Figure 9) $F(2, 52) = 7.23, p = .002$, and females (Figure 8), $F(2, 53) = 4.89, p = .01$ but no main effect of age. Of interest, there was a significant Drug x Age interaction for females, $F(2, 53) = 3.52, p = .037$, but not for males. $F(2, 52) < 1$. Outcomes from Figure 8 suggest that nicotine was anxiogenic at the lower .15 mg/kg dose for adolescent females but not for adult females. A subsequent interaction contrast including only female saline and .15 mg/kg animals revealed a significant Drug X Age interaction, $F(1, 37) = 4.89, p < .03$, with adolescent females showing significantly higher LES at the .15 mg/kg dose compared to saline, $F(1, 37) = 6.12$, whereas adult females treated with .15 mg/kg nicotine did not differ from saline controls, $F(1, 37) < 1$ (see Footnote 2). This latter outcome suggests that at some (lower) doses of nicotine, adolescent females show a vulnerability to the anxiety-producing effects of nicotine that adult females do not.

These results indicate, in contrast to what was predicted, adolescents did not display a
greater anxiogenic response to nicotine that adults. Furthermore, although the overall anxiogenic dose-response pattern was similar for adolescent and adult males, the pattern differed in adolescent versus adult females. This implies important neurodevelopmental changes in females occur from adolescence to adulthood that underlie females’ response to the anxiogenic effects of nicotine. In addition, adolescent females were sensitive to the anxiety-producing properties of moderate-dose (0.15 mg/kg) nicotine whereas adult females were tolerant to these effects.

**Conclusions**

This experiment successfully established the light-enhanced startle paradigm as a potential model of anxiety in adolescent animals. Collapsed across other factors, adolescents demonstrated light-enhanced startle (Figure 5) and did so in a similar fashion as would be expected in adults (Figure 4). Additionally, acute nicotine exposure in drug-naïve animals was shown to be anxiogenic. As predicted, females were found to generally express higher levels of light-enhanced startle in response to nicotine than males, but not without exceptions. Notably at the lower dose of nicotine, adult males, but not adult females, were sensitive to the anxiogenic effects of nicotine (Figures 6 and 7). It was also predicted that adolescents would display a higher overall change in startle amplitude than adults in response to nicotine. This, however, was not found to be the case. As evidenced by Figure 8 and Figure 9, such a pattern is not apparent. In limited circumstance, however, specifically at lower dose nicotine, adolescent females were more sensitive to the nicotine’s anxiogenic effects compared to adults (Figure 8).

Results of the present research have important implications relevant to human tobacco use. Because males displayed less elevated anxiety in response to acute nicotine exposure compared to females, this may indicate that males are more tolerant to the aversive anxiogenic effects of initial nicotine exposure, a pattern especially clear in adolescents (Figure 6). Therefore, males may be more likely to initiate a nicotine habit due to this lower relative aversion to the negative affects of nicotine on anxiety. Indeed, males account for four fifths of current smokers. Females, however,
account for a greater percentage of the population diagnosed with mental illness, revealing a complex interaction involving the effects of sex on nicotine addiction and psychiatric disease (Pigott, 1999). By the same logic, adolescents may be especially vulnerable in their potential to progress to physical dependence after initial smoking experiences because of their reduced sensitivity to the negative anxiety-producing effects of initial nicotine exposure (Natividad et al., 2010; O'Dell, 2009; Spear, 2000). This would be consistent with the unanticipated finding in this experiment that adolescents, with limited exception, showed a trend toward more resistance than adults to the anxiogenic effect of nicotine in light-enhanced startle.
References


Footnotes

1 Fewer adult animals were used in this experiment than adolescents for a practical reason. Among litters that were available for this research, it was decided to preferentially allocate animals to the adolescent test condition because of the relative lack of available data concerning LES in adolescent animals. This research is ongoing with the intention of increasing N in the adult conditions.

2 These specific mean comparisons are redundant with mean comparisons previously conducted on data illustrated in Figures 6 and 7. However, here they are conducted from interaction contrasts not previously reported and that use error terms specific to these contrasts which directly asses the effects of age.
Figure Captions

Figure 1. Elevated Plus Maze

Figure 2. Light-Dark Box

Figure 3. Light-Enhanced Startle Chamber

Figure 4. Mean raw peak startle for adult animals during Phase 1 and Phase 2 for Test Day 1 and Test Day 2 of the light-enhanced startle test (no drug exposure). Phase 1 and Phase 2 means are shown averaged across all other variables (dB amplitude, drug dose, sex). Brackets represent standard errors.

Figure 5. Mean raw peak startle for adult animals during Phase 1 and Phase 2 for Test Day 1 and Test Day 2 of the light-enhanced startle test (no drug exposure). Phase 1 and Phase 2 means are shown averaged across all other variables (dB amplitude, drug dose, sex). Brackets represent standard errors.

Figure 6. Mean percentage change scores for male and female adolescent animals as a function of drug dose during the critical Test Day 3 with nicotine. Brackets represent standard errors.

Figure 7. Mean percentage change scores for male and female adult animals as a function of drug dose during the critical Test Day 3 with nicotine. Brackets represent standard errors.
Figure 8. Mean percentage change scores for female adolescent and adult animals as a function of drug dose during the critical Test Day 3 with nicotine. Brackets represent standard errors.

Figure 9. Mean percentage change scores for male adolescent and adult animals as a function of drug dose during the critical Test Day 3 with nicotine. Brackets represent standard errors.
Figure 4: Adults
Day 1 & Day 2: all animals

Figure 5: Adolescents
Day 1 & Day 2: all animals
Figure 6: Adolescents
Day 3 Nicotine: Males vs. Females

Figure 7: Adults
Day 3 Nicotine: Males vs. Females
Figure 8: Females
Day 3 Nicotine: Adolescents vs. Adults

Figure 9: Males
Day 3 Nicotine: Adolescents vs. Adults