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# Light-Enhanced Startle as an Experimental Model of Withdrawal

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# Light-Enhanced Startle as an Experimental Model of Withdrawal

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by

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**Abstract**

Nicotine is extremely addictive, and the negative effects of withdrawal, such as increased anxiety, contribute to relapse. Nicotine produces significant changes in the extended amygdala, the brain regions responsible for regulating anxiety. Light-Enhanced Startle is an established method for assessing anxiety in animal models and is sensitive to drug manipulations. This thesis had two goals, Experiment 1 asked: can LES be used to investigate the anxiety of extended nicotine withdrawal following chronic nicotine administration in rats? We hypothesized that exposing rats to chronic nicotine would result in elevated LES during spontaneous withdrawal as compared to controls, and that this elevation in LES would decrease as withdrawal progressed. Adult Sprague-Dawley rats were given .40 mg/kg nicotine twice daily for 14 days, and were then tested in LES on withdrawal days 1, 3, and 5. Animals showed significantly increased anxiety on withdrawal day 1, and exhibited a pattern of decreasing but elevated LES on withdrawal days 3 and 5 consistent with our predictions. Experiment 2 built on Experiment 1, and asked: what effect will brief nicotine pre-exposure have on the anxiety of withdrawal following later administration? We hypothesized that briefly exposing animals to nicotine, followed by abstinence and then chronic exposure, would potentiate the anxiogenic effects of withdrawal, resulting in even further elevated LES during withdrawal. Adult rats were given 4 days of .40 mg/kg nicotine, followed by 10 days of abstinence, and then chronic nicotine and LES as described above. Surprisingly, animals in Experiment 2 displayed a general decrease in LES, indicating that nicotine pre-exposure has an anxiolytic effect during later withdrawal. These findings demonstrate that nicotine pre-exposure has a significant impact on how the brain responds to later nicotine, and this could be caused by a unique pattern of nAChR desensitization and upregulation resulting from pre-exposure.

## Light Enhanced Startle as an Experimental Model of Withdrawal

Cigarette smoking and tobacco usage in the United States is on the decline, yet 17% of adults still smoke cigarettes (CDC 'Current', 2015). Cigarette smoking and nicotine use is extremely addictive, and in a given one-year period over 50% of smokers will attempt to quit, while only 6% will actually be able to stop smoking (CDC 'Prevalence', 2015). Alternative tobacco products such as e-cigarettes have also increased in popularity in the past decade, and up to 20% of individuals who quit cigarettes in a given year continue to use electronic cigarettes containing nicotine (CDC 'Electronic', 2015). The adverse effects of nicotine withdrawal such as depression, physical pain, and increased anxiety are factors that promote relapse and prevent successful quit attempts (Vorvick, 2013). Nicotine withdrawal has also been shown to reduce brain reward function, another way in which the drug increases the chances of relapse (Epping-Jordan et al., 1998). Understanding the negative effects of withdrawal and when these effects are strongest is critical to enhancing the effectiveness of interventions and cessation efforts, and to progressing our knowledge about addiction by extending analysis of the effects of addiction beyond the period of drug exposure. Continued study of nicotine at both the molecular and behavioral levels of analysis remains important as long as nicotine is a common drug of abuse. Using an animal model of anxiety, the present research sought to develop an experimental model of nicotine withdrawal.

Nicotine's molecular target in the central nervous system is the nicotinic acetylcholine receptor (nAChR; Pistillo et al., 2014). Nicotine is an nAChR agonist, and activates nAChRs when it binds, mimicking the action of the endogenous neurotransmitter acetyl choline (ACh; Pistillo et al., 2014). NAChRs are widespread in the brain, and one area of particularly high nAChR density is the mesocorticolimbic system, which contains the brain regions responsible

for the maintenance of addiction. The mesocorticolimbic system includes the Ventral Tegmental Area (VTA), the Nucleus Accumbens (NAcc) and the ventral striatum, as well as the Pre-Frontal Cortex (PFC; Pistillo et al. 2014). Besides containing the neural circuits responsible for drug addiction, these regions also mediate processes such as motivation, decision making, and perceived value (Pistillo et al., 2014). NAChRs are pentameric channel proteins that are composed of a combination of 8 known alpha subunits and 3 known beta subunits, and receptors with different combinations of subunits are expressed in different brain regions (Pistillo et al., 2014). These different subtypes of nAChRs are linked to different functions and effects in the brain, and have also been suggested to play a role in regulating the plasticity of neural circuits, an activity that is crucial in the process of drug learning as well as addiction and withdrawal (Pistillo et al., 2014).

Several different specific subtypes of nAChRs are implicated as mediators of both general anxiety and anxiety produced by addiction and withdrawal. Meyers, Loetz, and Marks (2015), for example, have shown that nAChRs subtypes containing a $\beta_4$ subunit are critically important in the molecular process of the anxiety that follows nicotine administration. Genetically deleting the $\beta_4$ receptor subunit in mice resulted in an almost complete absence of the somatic signs of nicotine withdrawal (Meyers et al., 2015). Chronic nicotine administration has distinct molecular effects, one of which is an upregulation in levels of nAChRs (Pistillo et al., 2014). In individuals that are repeatedly exposed to high doses of nicotine, such as chronic smokers, the brain responds to the near continuous activation of nAChRs by increasing the density of these receptors on neuronal membranes (Quick & Lester, 2002). This up-regulation the in amount of receptors causes a depression in nAChR signaling, which can also be described colloquially as nAChRs having a “thirst” for ACh when nicotine levels decrease in the brain

during withdrawal (Quick & Lester, 2002). When nicotine levels in the brain are decreased, such as in smoking cessation, this functional depletion of ACh is associated with withdrawal (Quick & Lester, 2002).

Animal models of anxiety have been used to study how withdrawal from nicotine can affect stress and anxiety in people, and how these events can contribute to addiction. One recognized behavioral model of anxiety is the elevated plus maze (EPM). The EPM is used as an assay for behavioral anxiety in rodents, in which animals traverse "open" versus "closed" arms of a simple plus maze. Closed arms are enclosed with typically tall walls and possibly a ceiling whereas Open arms are typically flat platforms that expose the animals to light and open space of the test room. Rodents have a natural preference for the relative safety of closed arms. Anxiety is determined as a function of how much time the animal spends in the open arms or the number of times it enters an open arm. More time spent on or more entries into open arms is taken as a less anxious state; more time spent or more entries into closed arms is taken as a more anxious state. An additional common assay for behavioral anxiety is the Open Field test, in which rodents are placed in an enclosed area. Anxious behavior is defined as more time spent against walls or in corners as compared to the open central area. Both the EPM and Open Field test rely on the fact that rodents are nocturnal, and brightly lit and open spaces are known to be naturally aversive to them. Torres et al. (2013) demonstrated that following 14-days of chronic nicotine exposure administered via subcutaneous osmotic mini-pump, rats displayed increased anxiety measured in EPM (increased closed arm entry) and Open Field test (increased time spent in corners) during acute nicotine withdrawal relative to controls. Additionally, acute withdrawal was associated with elevated levels of corticotropin-releasing factor (CRF, a stress hormone) consistent with the anxiety-inducing effects of withdrawal (Torres et al., 2013).

Another validated animal model of anxiety is known as Light-Enhanced Startle (LES). The LES paradigm has been used to examine the neuroanatomical and pharmacological basis of anxiety (Walker & Davis, 1997). In LES, rats are exposed, either in the presence of a bright light or in darkness, to brief bursts of white noise that elicit an acoustic startle reflex (Walker & Davis, 1997). Rodents, normally nocturnal, find bright light naturally anxiety producing and the magnitude of the noise-elicited startle reflex is larger in the presence of a bright light as compared to darkness (Walker & Davis, 1997). A key component of the LES paradigm is that it models un-conditioned (unlearned) anxiety, as opposed to fear based on prior learning (Walker & Davis, 1997). This means that LES does not extinguish, and that the same subjects can be repeatedly tested over time (Walker & Davis, 1997). In addition, LES has been shown to be susceptible to drug manipulation, both anxiogenic and anxiolytic, making it ideal for the investigation of nicotine drug effects on anxiety during extended withdrawal. (Walker & Davis, 1997).

In the only currently published paper examining the effect of nicotine withdrawal in LES, Jonkman et al. (2008) exposed rats to 28 days nicotine using an osmotic minipump which doses nicotine continuously over each 24 hr period while the pumps are active (in principle like a nicotine patch). Twenty-four hours after the nicotine dosing period ended (during immediate, acute, withdrawal) rats were tested in LES (Jonkman et al., 2008). Nicotine treated rats showed greater LES than control rats, although Jonkman et al. (2008) tested only once precluding assessment of the time course of anxiety during withdrawal.

In a related study, Engelmann, Radke, and Gewirtz (2009) exposed rats to 14 days of intraperitoneal (I.P.) injections of nicotine followed by testing in an acoustic startle paradigm in order to measure anxiety effects of nicotine withdrawal. Engelmann et al.'s (2009) use of I.P. as

opposed to subcutaneous osmotic nicotine delivery may better model human nicotine intake patterns (smoking) given that I.P. delivery (nicotine injection), like cigarette smoking, causes a rapid rise in nicotine brain and blood levels, as well as a period of abstinence that occurs between each I.P. nicotine injection, similar to cigarette use. Engelmann et al.'s (2009) method also differed from that of Jonkman et al. (2008) by examining the effect of nicotine withdrawal on baseline startle responding produced by a startle-eliciting noise per se, not the effect of nicotine withdrawal on augmentation of LES. Engelmann et al. (2009) found that startle responding was not elevated during acute immediate withdrawal 1 day following nicotine cessation but was elevated during withdrawal 7 and 15 days following nicotine cessation. Additionally, withdrawal-induced anxiety (elevated startle) was found to be reduced either by re-exposure to nicotine or the nicotinic antagonist clonidine, implicating a role of nAChRs in withdrawal-mediated alterations in startle behavior.

*Research Hypothesis and Predictions*

The present experiments were behavioral in focus and empirically motivated. Some research, albeit limited, suggests startle paradigms in general (i.e., Engelmann et al., 2009) and LES in particular (i.e., Jonkman et al., 2008) may be a useful model for studying anxiety associated with nicotine withdrawal. The two experiments reported here were modeled after but extended those of Jonkman et al., (2008) and Engelmann et al. (2009). In Experiment 1, rats were exposed to 14 days of I.P. nicotine or saline followed by behavioral testing in LES 1, 3, and 5 days after the start of the withdrawal period. The question of Experiment 1 was whether nicotine treated animals would display elevated LES compared to saline treated animals. We hypothesized that animals exposed to chronic nicotine would display increased anxiety during withdrawal, and that these effects would decrease over the course of withdrawal.

Experiment 2 was intended to replicate and potentially enhance findings from Experiment 1. In Experiment 2, rats were again exposed to 14 days of I.P. nicotine or saline followed by LES testing on withdrawal days 1, 3, and 5. However in Experiment 2, the 2-week nicotine exposure period was preceded 10 days earlier by a brief 4-day pre-exposure to nicotine shown in other research (Arnold et al., 2003) to sensitize nicotine's effect on the brain (specifically, ACh release). We hypothesized that animals exposed to an additional pretreatment of nicotine as well as chronic nicotine would display even stronger anxiety during spontaneous withdrawal, and that these effects would decrease over the course of withdrawal. The logic of Experiment 2 will be discussed in more detail later.

In both of the present experiments, interest was in assessing anxiety associated with withdrawal measured in LES. Notably, Jonkman et al. (2008) and Engelmann et al. (2009) employed only male subjects precluding analysis of sex effects. The present research included both males and females to assess possible sex effects in withdrawal-associated anxiety. If LES is a useful model for examining withdrawal-produced anxiety, LES should be enhanced during the withdrawal period in previously nicotine treated animals compared to controls.

## Experiment 1

The goal of Experiment 1 was to assess whether the LES paradigm is sensitive to anxiety associated with withdrawal. Rats were exposed to 2x/daily I.P. nicotine (0.40 mg/kg) or saline for 14 days. Following nicotine exposure animals were tested in acute withdrawal 1, 3, and 5 days following nicotine cessation. If LES is sensitive to anxiety associated with withdrawal, the magnitude of LES should be higher in previously nicotine treated animals compared to previously saline treated animals.

## Method

*Subjects*

Forty-eight Sprague-Dawley (24 male and 24 female) rats were obtained from the breeding colony of the Department of Psychology at The College of William & Mary. Animals were weaned on PD21 and gang housed in plastic cages by litter and were moved to individual plastic cages 2 days prior to first injection. Rats were under a 14 hr/10 hr light-dark cycle and were given access to water and food *ad libitum.* All animal care procedures were carried out in accordance with approved IACUC protocols.

*Apparatus*

Light-enhanced startle testing occurred in three identical small startle cages. The startle cages served as functional restraint cages (Coulbourn Instruments, Model E05-20) 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 black 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 11cm x 9.5 cm (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 52cm 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 5-lb

maximum output transducer platform (Coulbourn Instruments, Model E45-15) 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 20-W (rated at 1300 lumens), General Electric compact fluorescent light bulb could be turned on to provide bright illumination of the chamber. 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.

*Procedure*

Rats were counterbalanced into 2 experimental drug conditions, with respect to weight, litter, and sex. The two drug conditions (*n*s = 12 within each drug and sex subgroup) consisted of high dose of nicotine (0.40 mg/kg) or vehicle control (saline).

*Nicotine Exposure*. Beginning on approximately PD85 rats were exposed 14 days of intraperitoneal (I.P.) nicotine or saline injections administered 2x/daily, depending on drug designation. Injections occurred once in the morning (approximately 0830) and once in the early afternoon (approximately 1430). Nicotine was nicotine tartrate dissolved in saline and titrated to a pH of 7.4. Rats were weighed prior to each injection and received 1 ml/kg mixed nicotine solution based on body weight.

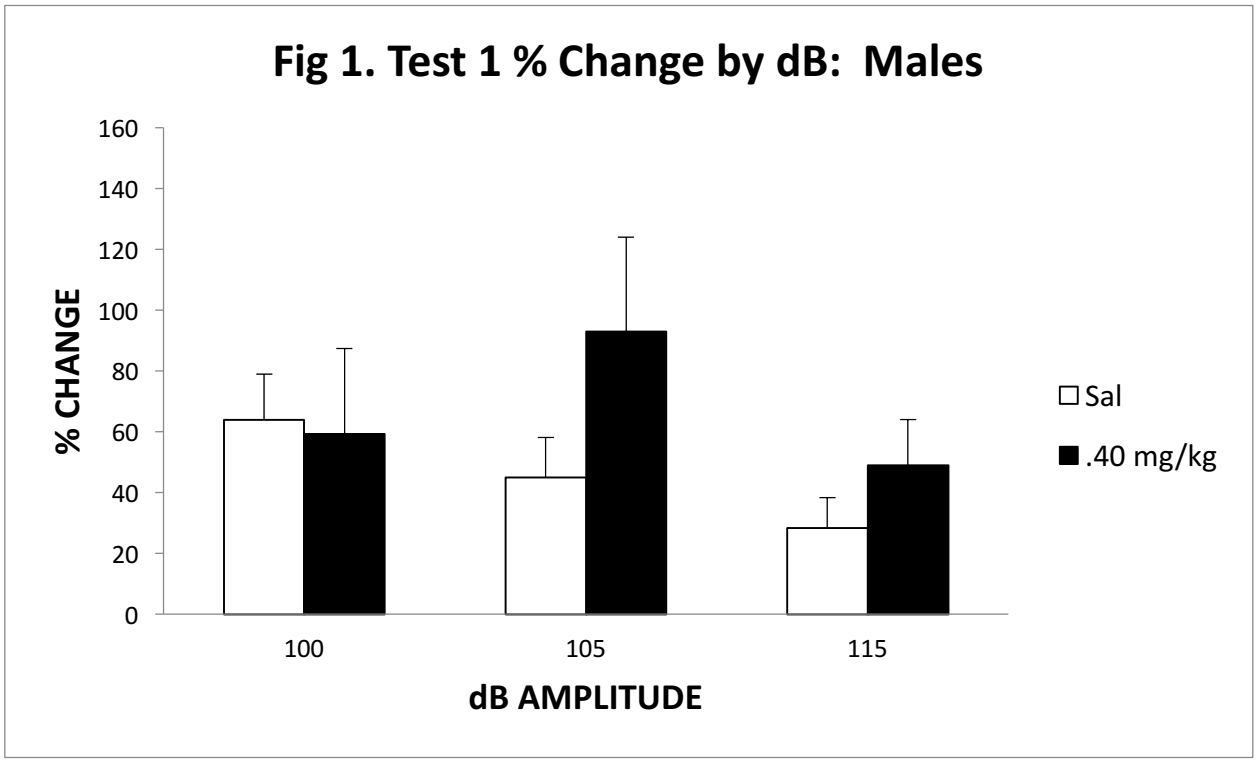
*LES Test Procedure*. Behavioral testing in LES was initiated 24 hours following the last nicotine injection. Following the last nicotine exposure, LES testing occurred at 1 day (24 hrs), 3 days (72 hrs) and 5 days (120 hrs) during the withdrawal period. Each LES test session was comprised of two 20-min phases with each phase separated by a 5-minute rest period. During Phase 1 rats were placed in the startle chamber and after 600 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]). Phase 1 was conducted in the dark with no chamber illumination. 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 except that Phase 2 was conducted in the presence of a bright (20-W compact fluorescent) light. 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 only by the absence (Phase 1) or presence (Phase 2) of the bright light. Data from LES testing were expressed as a percentage of change score computed as: ([Ph2 (Light) startle mean – Phase 1 (Dark) startle mean] / [Phase 1 startle mean]) *100.

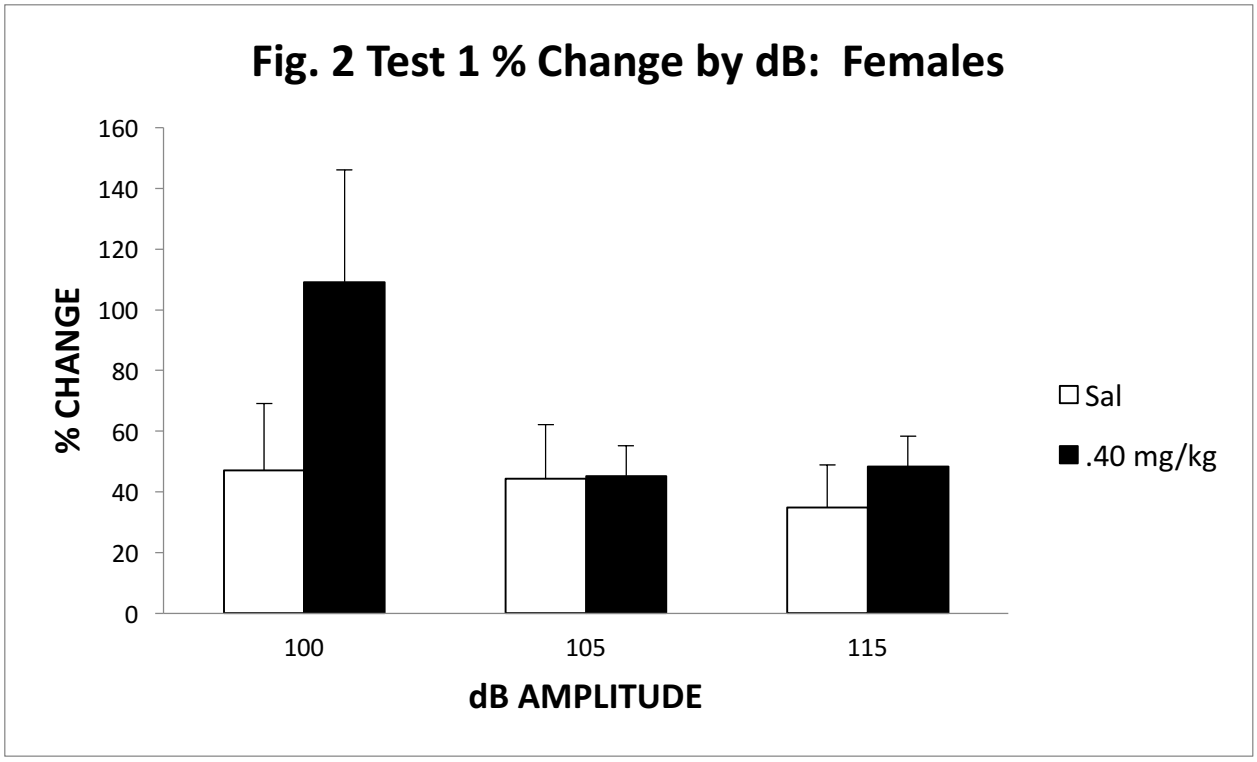
## Results and Discussion

Data from the three LES tests during nicotine withdrawal are presented in Figures 1-6. Overall, the data suggest that there was an elevation in the magnitude of LES in nicotine treated groups compared to non-nicotine treated groups in both males and females, although this elevation varied with sex and dB amplitude of startle pulse across tests. During immediate, spontaneous withdrawal on Test 1 nicotine treated males had markedly higher LES at the 105 dB amplitude (Figure 1) and nicotine treated females had higher LES at the 100 dB amplitude (Figure 2). Numerically, the pattern for males was generally consistent across testing with nicotine treated males demonstrating higher LES at 105 and 115 dB amplitudes on Test 1 (Figure 1), Test 2 (Figure 3) and Test 3 (Figure 6). Females also showed a generally consistent trend with numerically greater LES in nicotine treated animals at the 100 dB amplitude on Test 1 (Figure 2), Test 2 (Figure 4) and Test 3 (Figure 6), although that elevation was fairly small on the latter two tests. Statistical analyses revealed significant drug effects only on Test 1.

A Test (Test 1, Test 2, Test 3) X dB (100dB, 110dB, 115dB) X Sex (Male, Female) X Drug (Saline, 0.40 mg/kg nicotine) Mixed ANOVA conducted on percentage change scores from Experiment 1 revealed a significant dB X Sex X Drug interaction, $F(2, 176) = 7.3$, $p < .001$, as well as a Test X dB interaction, $F(4, 176) = 2.64$, $p < .05$. Planned comparisons were conducted on group means from each test separately in order to maintain consistency of analysis with Experiment 2 and significant drug effects were found only in Test 1. The source of the dB X Sex X Drug interaction appeared to be that nicotine treated males on Test 1 had significantly higher percentage change scores than saline treated males only for the 105 dB amplitude, $F(1, 176) = 10.23$, $p < .05$, whereas nicotine treated females had significantly higher percentage change

scores compared to saline treated females only for the 100dB amplitude, $F(1, 176) = 6.09$, $p < .05$.



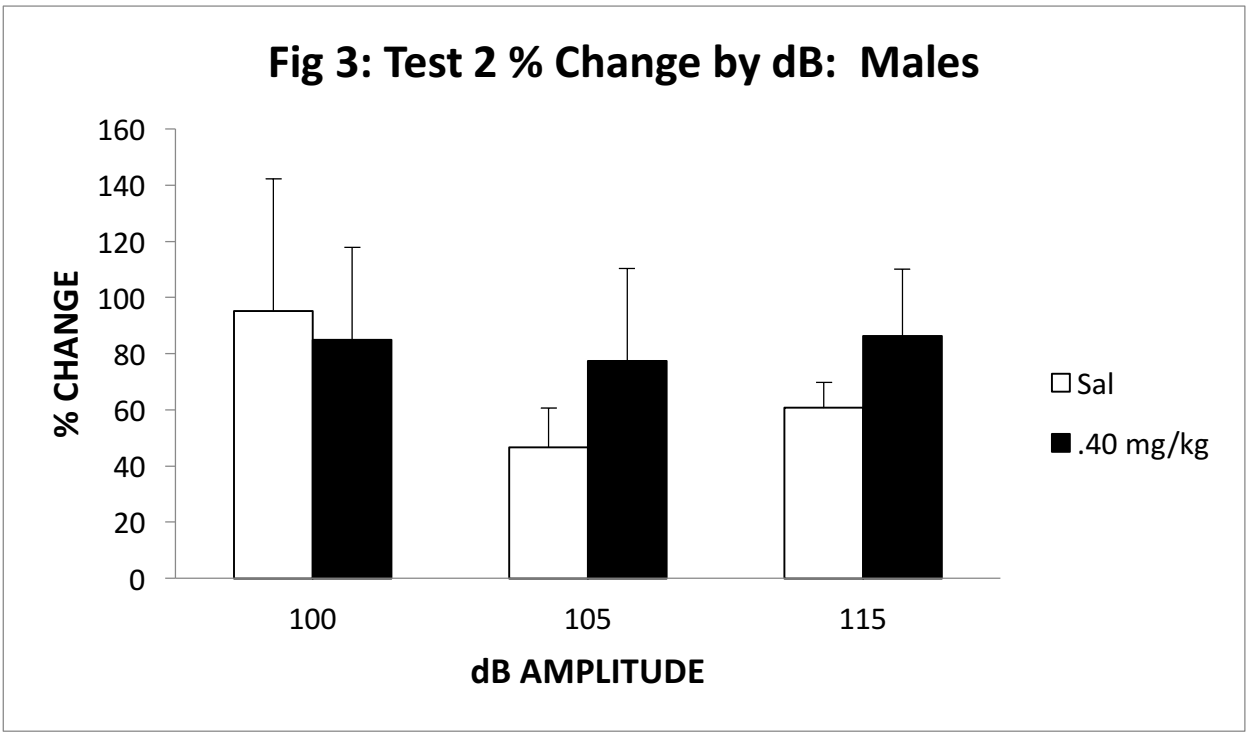


Fig 3: Test 2 % Change by dB: Males
% CHANGE
160
140
120
100
80
60
40
20
0
100
105
115
dB AMPLITUDE
Sal
.40 mg/kg

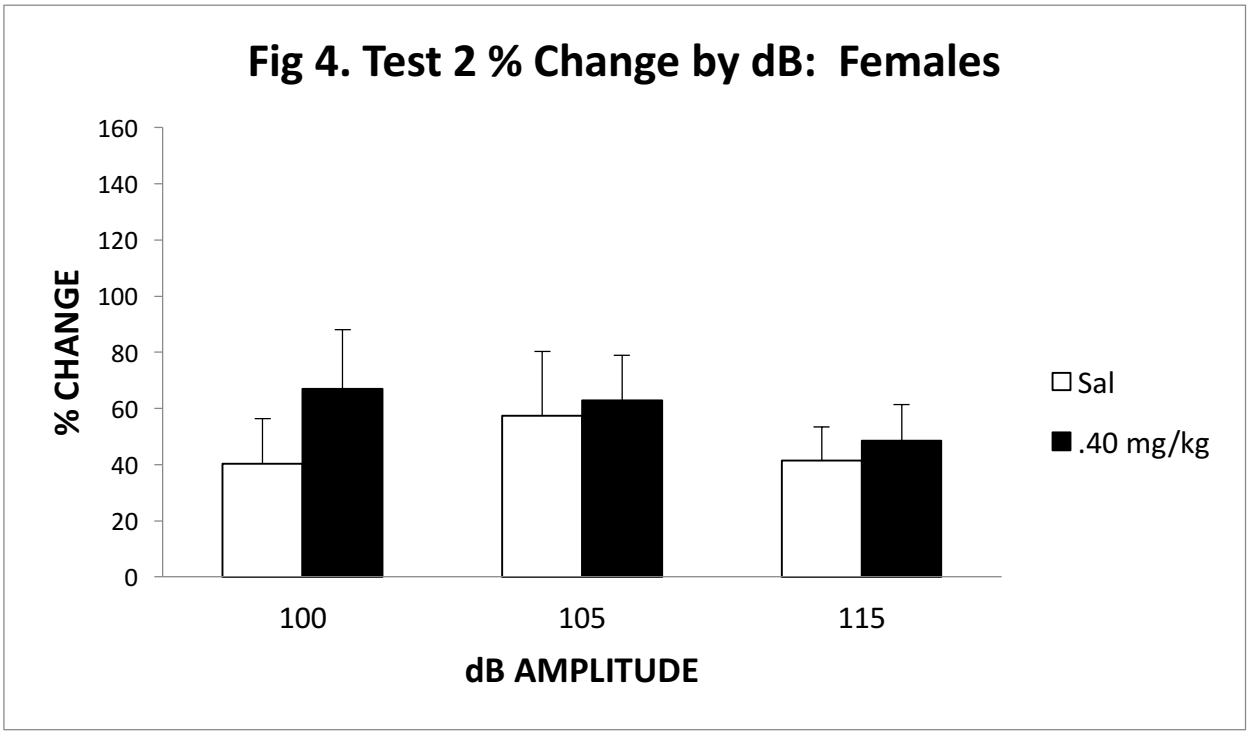


Fig 4. Test 2 % Change by dB: Females
% CHANGE
160
140
120
100
80
60
40
20
0
100
105
115
dB AMPLITUDE
Sal
.40 mg/kg

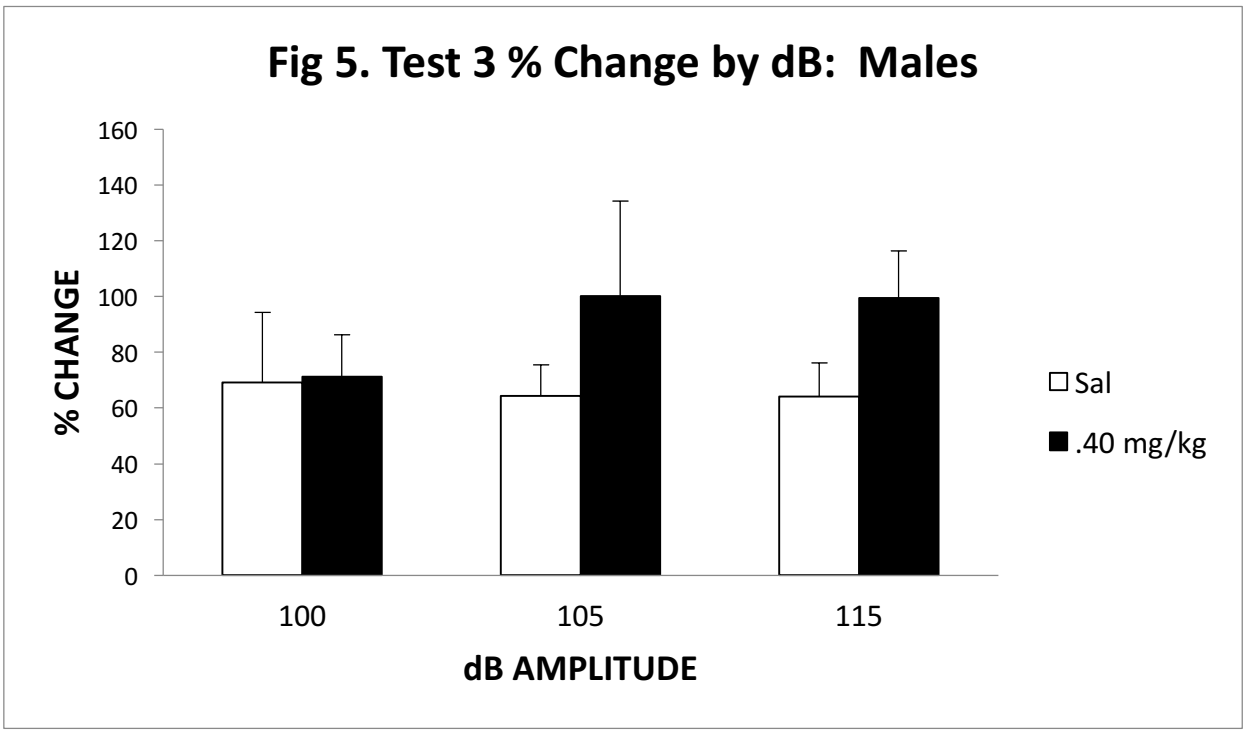


Fig 5. Test 3 % Change by dB: Males
% CHANGE
160
140
120
100
80
60
40
20
0
100
105
115
dB AMPLITUDE
Sal
.40 mg/kg

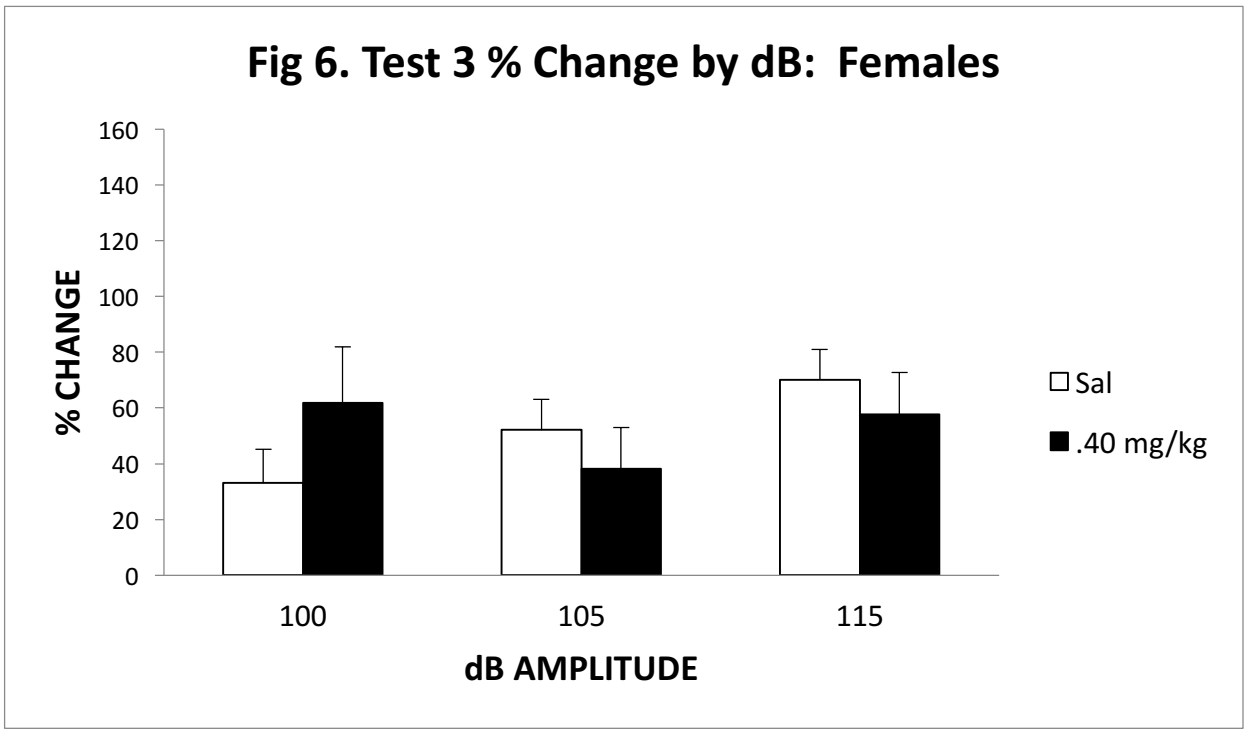


Fig 6. Test 3 % Change by dB: Females
% CHANGE
160
140
120
100
80
60
40
20
0
100
105
115
dB AMPLITUDE
Sal
.40 mg/kg

## Experiment 2

Findings from Experiment 1 provided qualified support suggesting LES may be a useful tool for measuring anxiety during acute withdrawal from nicotine. Experiment 1 also suggested that anxiety may be highest during immediate withdrawal, namely, early in the withdrawal period. Arnold et al. (2003) demonstrated that brief, 4-day, exposure to nicotine sensitized nicotine's ACh-releasing effects when nicotine was later encountered. Arnold et al. (2003) exposed adult rats to 4 days of I.P. nicotine then measured the ability of subsequent nicotine re-exposure to promote ACh release. ACh release was observed to be elevated, or sensitized, in animals pre-exposed to nicotine compared to non pre-exposed animals (Arnold et al., 2003). Arnold et al. (2003) further observed that this 'sensitization effect' was reduced to control levels by 10 days following the 4-day sensitizing nicotine pre-exposure.

The goal of Experiment 2 was to replicate and potentially enhance the withdrawal-induced elevation of LES seen in Experiment 1. Specifically, Experiment 2 examined whether a brief 4-day preexposure to nicotine would sensitize the anxiety-inducing effects of nicotine withdrawal as a result of later protracted 14-day nicotine exposure – namely, when nicotine was later encountered. Rats were exposed to 4 days of I.P. nicotine followed by 10 days of abstinence modelling the sensitization protocol of Arnold et al. (2003). The 10-day abstinence period was then followed by 14 days of nicotine treatment identical to that of Experiment 1. The nicotine exposure sequence in Experiment 2 was intended to do more than simply add four more days of nicotine. Rather, the question was whether a brief early exposure to nicotine that would be less likely to produce full addictive dependence (yet known to have sensitizing effects) would, *later and after a period of abstinence*, enhance the effect of nicotine-induced dependence produced by chronic nicotine, and therefore withdrawal. Arnold et al. (2003) reported that the sensitizing

effects of brief 4-day nicotine exposure (measured in nicotine-induced Ach release) reduced to control levels after 10 days following the last nicotine preexposure. Thus, 10 days abstinence between 4-day nicotine preexposure and 14-day nicotine exposure in Experiment 2 was intended to allow the immediate sensitizing effects of nicotine to fully dissipate while at the same time permitting the assessment of the longer-lasting impact of nicotine preexposure on withdrawal produced by more chronic nicotine intake. If nicotine preexposure enhances the brain's response to nicotine when later encountered (and by inference its addictive potential), then nicotine pretreatment might promote a stronger withdrawal-associated elevation in LES compared to that of Experiment 1. Due to the practical issue of animal availability, the logical control group that did not receive 4-day nicotine pretreatment but did receive 14-day nicotine exposure was not included. Experiment 2 was essentially a replication of Experiment 1 with the addition of 4-day nicotine pretreatment.

## Method

### *Subjects*

Forty-eight Sprague-Dawley (24 male and 24 female) rats served as subjects and were housed and maintained as in Experiment 1. One female rat became ill during the course of the experiment and was eliminated.

### *Apparatus*

The apparatus was as in Experiment 1.

### *Procedure*

Rats were counterbalanced into two groups designated by drug condition, counterbalanced with respect to weight, litter, and sex (ns ≈ 12 within each drug and sex

subgroup). As in Experiment 1 there were two drug conditions: high dose of nicotine (0.40 mg/kg) or vehicle control (saline).

*Nicotine Exposure.* Rats were exposed to 4 consecutive days of 2x/daily nicotine or saline injections beginning approximately on PD 80 followed by 10 days of nicotine abstinence, which was then followed by another 14 consecutive days of 2x/daily nicotine or saline injections beginning approximately on PD 95 otherwise identical to that of Experiment 1.

*LES Test Procedure.* LES testing occurred as in Experiment 1 with LES testing on Withdrawal Days 1, 3, and 5.

## Results and Discussion

In contrast to the expected enhancement of LES following nicotine preexposure coupled with 2-week chronic nicotine exposure relative to saline controls, there was no evidence of elevated LES in nicotine treated animals during withdrawal testing (Test 1, Figure 7; Test 2, Figure 8; Test 3, Figure 9). As seen in Figure 9 (Test 3), a surprising outcome and contrary to prediction, nicotine treated females displayed lower LES compared to saline treated females.

A Test (Test 1, Test 2, Test 3) X dB (100dB, 110dB, 115dB) X Sex (Male, Female) X Drug (Saline, 0.40 mg/kg nicotine) Mixed ANOVA conducted on percentage change scores from Experiment 2 revealed a significant Test X Sex X Drug interaction, $F(2, 172) = 3.03$, $p < .05$, as well as a Test X Sex interaction, $F(2, 172) = 2.64$, $p < .05$. Subsequent comparisons averaged over dB were conducted on data from each test day separately in order to evaluate hypotheses about the effect of nicotine on anxiety measured in LES. On Test 3 nicotine treated females but not males showed significantly *lower* percentage change scores compared to saline treated animals $F(1, 172) = 9.71$, $p < .01$, whereas percentage change scores did not differ across drug conditions in either males females on Test 1 or Test 2.

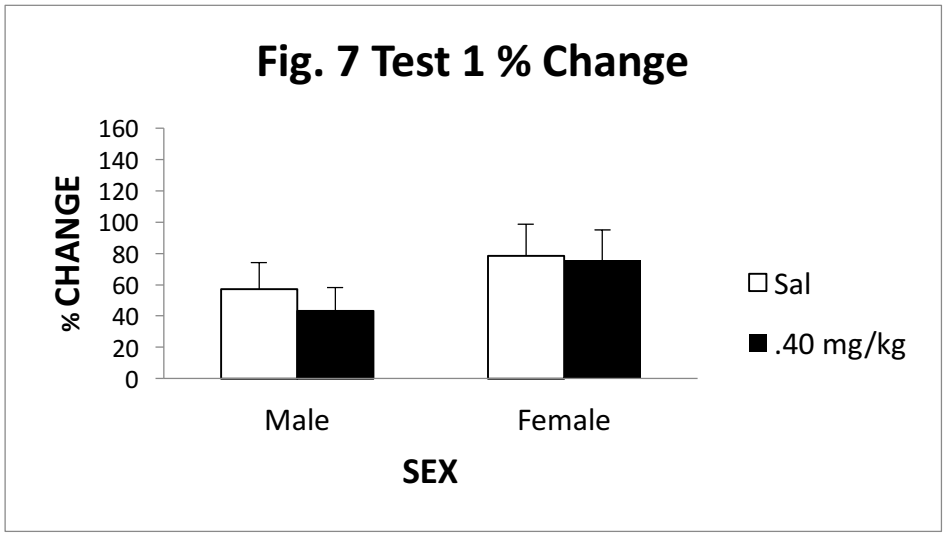


Fig. 7 Test 1 % Change
% CHANGE
160
140
120
100
80
60
40
20
0
Male
Female
SEX
Sal
.40 mg/kg

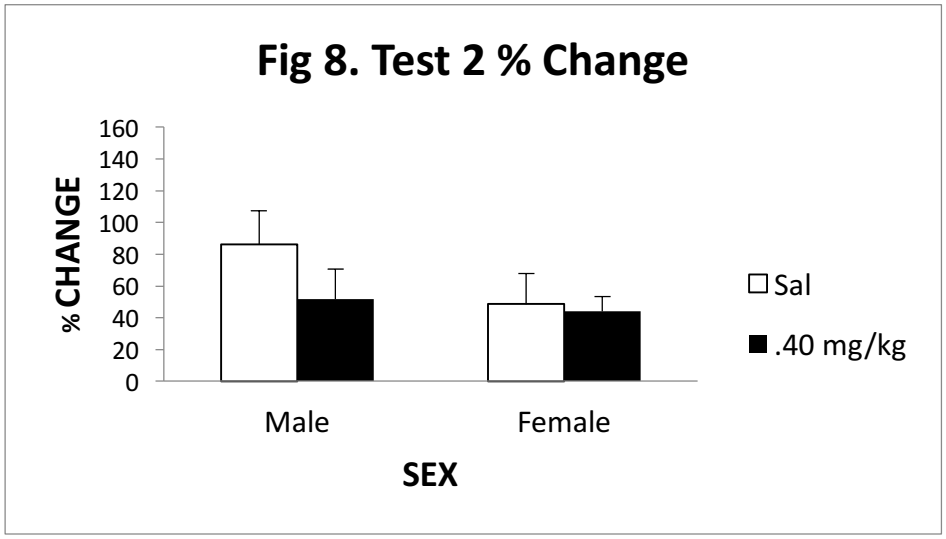


Fig 8. Test 2 % Change
% CHANGE
160
140
120
100
80
60
40
20
0
Male
Female
SEX
Sal
.40 mg/kg


Fig 9. Test 3 % Change
% CHANGE
160
140
120
100
80
60
40
20
0
Male
Female
SEX
Sal
.40 mg/kg

## General Discussion

In Experiment 1, we predicted that exposure to chronic nicotine would result in increased levels of anxiety during withdrawal as indicated by an increase in LES, and that this increase would be greatest early in the withdrawal period. Our prediction was supported by the significantly elevated LES observed on Test 1 in both nicotine treated males and females, as well as the subsequent trend of decreasing, but elevated LES during the time-course of withdrawal in nicotine treated animals. These results are consistent with other research that shows that exposure to chronic nicotine significantly increases anxiety during spontaneous withdrawal (Jonkman et al., 2008; Torres et al., 2013) and that these effects can last beyond the initial withdrawal period (Englemann et al., 2009).

In the brain, addiction is characterized by a drastic increase in dopaminergic signaling resulting in a behavioral shift towards drug seeking behavior (Paolini & De Biasi, 2011). Like many other common drugs of abuse, exposure to nicotine changes dopamine (DA) utilization in the VTA, PFC, NAcc, and dorsolateral striatum (George et al., 1998; Paolini & De Biasi, 2011). During withdrawal from nicotine, DA signaling is greatly decreased in the mesocorticolimbic system including the NAcc, and this decrease in the NAcc is primary cause of withdrawal symptoms (Paolini & De Biasi, 2011). The key component of withdrawal addressed in the present research is innate anxiety, and the Bed Nucleus of the Stria Terminalis (BNST) is critically implicated as a brain region responsible for modulating this behavior (Davis et al., 2010). Additionally, the BNST, outer shell of the NAcc, and the Central Nucleus of the Amygdala (CeA) together make up a region known as the extended amygdala, a superstructure of interconnected brain regions responsible for mediating anxiety behaviors in the brain and that is incredibly sensitive to nicotine withdrawal (Marcinkeiwcz et al., 2009). Marcinkeiwcz et al.

(2009) demonstrated that nicotine withdrawal in the extended amygdala is associated with a significant increase in tissue levels of the stress-marker Corticotrophin Releasing Factor (CRF), and that this increase is responsible for the negative affective behaviors associated with nicotine withdrawal.

These findings help us form a hypothesis of how chronic nicotine exposure results in significant brain changes that translate directly to increased innate anxiety during spontaneous withdrawal as seen in our results. Following cessation of chronic nicotine, the brain regions in the extended amygdala experience a significant increase in activity due to the sudden increase in excitatory signaling. In this state the brain's level of innate anxiety is increased, and its ability to handle stressful environmental stimuli is severely compromised. We hypothesize that females have a lower threshold and ceiling for stressful stimuli that can elicit a behavioral response, and this supported by our findings that females exhibited elevated LES at lower dB amplitudes as compared to males. Male rats exhibited LES at consistently higher dB amplitudes compared to females, suggesting a differential pattern of activity in brain regions of the extended amygdala. The withdrawal process is plastic, and over the course of withdrawal normal nAChR activation is slowly restored and levels of anxiety and ability to respond to stressful stimuli move towards baseline (Giniatullin et al., 2005). This is confirmed by our observation that the strongest enhancement in both males and females was on Test day 1. We predict that a future experiment investigating activation of the extended amygdala during spontaneous nicotine withdrawal using EEG or fMRI would see differential activation in females compared to males, and that activation in both sexes would decrease over time similarly to the results we obtained using LES as a behavioral assay. Additionally, we predict that levels of biological stress indicators such as CRF

would be elevated in these brain areas, and that as withdrawal proceeds levels of CRF would decrease.

In Experiment 2, we predicted that pre-exposing animals to nicotine would enhance the anxiety of withdrawal following later chronic drug exposure. This prediction was based on research indicating that nicotine sensitization produces significant changes to both cholinergic and dopaminergic signaling in the brain which has implications for innate anxiety and withdrawal (Arnold et al., 2003, George et al., 1998). Our actual results were surprising, and do not directly support this prediction. What we expected was a pattern of results similar to Experiment 1, but with an overall increased magnitude of LES in nicotine groups indicating an enhancement from preexposure. Our results instead indicate that nicotine sensitization results in a pattern of reduced behavioral anxiety in animals, and this effect can be temporally distal or proximal depending on sex. Nicotine treated males exhibited a pattern of decreased LES on Test days 1 and 2 but not on Test day 3. Drug treated females exhibited no difference in LES on Test days 1 and 2 compared to controls, but showed significantly decreased LES on Test 3.

The significant decrease in LES observed in females on Test day 3 deserves further qualification. When the magnitude of the % change score measure used for analysis is compared across all three tests, it is clear that the nicotine treated females did not exhibit a significant change in LES, rather the statistically significant difference is due to *saline* treated females exhibiting a significant *increase* in LES on Test day 3 (Figure 9). This is surprising and unexpected, and it should be noted that it is a possibility that this data is simply erroneous, caused either by pure chance or by test day abnormalities in experiment running, i.e. human error. However, we also must also consider the scenario in which the data are correct, and represent a unique pattern of behavior. This being the case, we hypothesize that a differential

pattern of nAChR desensitization and regulation triggered by the additional nicotine pre-exposure and abstinence period is responsible for these results.

It is known that nAChRs become desensitized to ACh and other agonists, such as nicotine, after repeated activation, and that this desensitization results in a decrease in excitatory signaling in nAChR dense brain regions (Giniatullin et al., 2005, Quick & Lester, 2002). Furthermore, nicotine has been shown to differentially desensitize nAChRs compared to endogenous ACh such that certain subtypes of nAChRs take a longer time to re-sensitize after exposure to nicotine compared to other agonists (Giniatullin et al., 2005). Maintained nAChR desensitization is proposed as one way in which withdrawal symptoms are actually relieved in some cases (Giniatullin et al., 2005). Along with nAChR desensitization, chronic nicotine exposure is also associated with an upregulation in nAChR density on neuronal surfaces (Quick & Lester, 2002). Some nAChR subtypes have a higher affinity for nicotine compared to other ACh agonists, and the upregulation of high affinity nAChRs can last long past the period of actual nicotine exposure (Giniatullin et al., 2005). Brief nicotine exposure is also known to cause long lasting morphological changes in the NAcc (Brown & Kolb, 2001).

We propose that the unexpected LES results seen in nicotine treated animals in Experiment 2 are due to differential nAChR desensitization and regulation compared to drug groups in Experiment 1, and that this difference was directly caused by the 4 days of nicotine pre-exposure and additional abstinence period in Experiment 2. We hypothesize that during the 4 days of nicotine exposure in Experiment 2, nAChRs in drug treated animals were desensitized and up-regulated, and that these changes persisted to some degree through the 10 day abstinence period. When animals were then exposed to the 14 days of chronic nicotine and later spontaneous withdrawal, the pre-exposed animals were primed for even further desensitization

and upregulation of high affinity nAChRs in brain regions critical in mediating anxiety such as the NAcc, BNST, and amygdala.

It is possible that during spontaneous withdrawal in Experiment 2, the pattern of desensitization of high affinity nAChRs in drug treated animals was stronger in magnitude and durability compared to drug treated animals in Experiment 1, resulting in the absence of immediate anxiogenic effects of nicotine withdrawal because nAChRs were still in a desensitized state. We propose that the added days of nicotine pre-exposure worked to keep nAChR signaling relatively stable and unaffected by external stimuli following cessation of nicotine administration. This is supported by the pattern of decreased LES seen in nicotine treated males in Test 1 and 2, which can be interpreted as a decrease in baseline startle response compared to the saline groups. Withdrawal anxiety occurs with some level of neuronal activity and during the process of nAChR re-sensitization, which we propose was absent in animals in Experiment 2. Durable differences in nAChR desensitization and regulation could also explain the perceived decrease in LES seen in drug treated females on Test 3. We propose that these results should be interpreted as decreased baseline responding to startle stimuli compared to controls due a durable increase in the desensitization and upregulation of high affinity nAChRs.

We hypothesize that the additional nicotine pre-exposure in Experiment 2 delayed the anxiogenic effects of spontaneous nicotine withdrawal. We hypothesize that if LES testing were conducted further along in the time-course of withdrawal following the nicotine administration schedule in Experiment 2, that a pattern of elevated LES similar to Experiment 1 would occur in drug treated animals. To determine whether the observed anxiolytic pattern in Experiment 2 was due to differential nAChR responses to nicotine, methods involving receptor density imaging and ligand-receptor interactions would need to be used. We predict that if such a study were

conducted comparing drug treated animals from Experiment 1 and 2, animals from Experiment 2 would display significantly increased nAChR desensitization and upregulation, and that these features would last past the initial period of withdrawal.

In summary, spontaneous withdrawal from chronic nicotine results in an increase in excitatory ACh and DA signaling in the NAcc, BNST, and Amygdala. These brain regions are critical in the circuitry of the brains innate level of anxiety as well as processing stressful environmental stimuli. Chronic nicotine administration results in the desensitization and upregulation of high affinity nAChRs in these brain regions, and this process is reversed during normal withdrawal. A period of brief nicotine pre-exposure and subsequent abstinence strongly attenuates the response of nAChRs to nicotine, resulting in changes that persist past the initial period of withdrawal following chronic administration. We propose that the attenuation of nAChRs' response to nicotine manifests as decreased baseline responding to stressful environmental stimuli, and that this response is subject to sex differences in the brain.

## Conclusion

One major goal of this project was to assess the viability of LES as a tool for measuring the anxiety produced during spontaneous withdrawal from nicotine. To that end this research was successful, results from Experiment 1 illustrate how LES detected anxiogenic behavior in nicotine treated animals during immediate spontaneous withdrawal, as well as a trend of decreasing anxiety as withdrawal progresses. Experiment 2 sought to explore how pre-exposing animals to nicotine altered anxiety during later withdrawal following chronic drug administration. Experiment 2 yielded surprising results, namely that pre-exposing animals to nicotine manifests as anxiolytic effects during withdrawal. One possible explanation for these results asserts that the schedule of drug administration used in Experiment 2 creates a unique

pattern of nAChR desensitization and up-regulation in brain areas critical in anxiety and withdrawal, and this results in reduced behavioral anxiety during the first 5 days of spontaneous withdrawal.

These results provide an excellent experimental question for future experiments involving both behavioral and molecular methods. Future research should further explore how brief exposure to nicotine interacts with later chronic exposure, both in terms of behavioral anxiety during administration and subsequent withdrawal and in terms of nAChR desensitization and regulation. Our results indicate that LES is a valid tool for assessing behavioral anxiety during both initial and extended drug withdrawal, however more research is needed to fully understand to what nature of experimental manipulations LES is sensitive. Our results from Experiment 2 perfectly set up further exploration of how nAChR sensitization and regulation relates directly to anxiety of withdrawal in the context of nicotine addiction. While the molecular mechanism of withdrawal and sensitization are well understood, nicotine produces interesting and complex effects depending on the schedule of drug administration, and these concepts deserve further study using advanced molecular techniques. Experiments replicating our methods with the addition of specific nAChR subtype knockout models, receptor imaging, or functional brain imaging would help create a more holistic understand of the anxiety of nicotine withdrawal and the effects of nicotine pre-exposure.

The current research also represents a meaningful contribution to the study of nicotine as a drug of abuse and the adverse effects that nicotine has on the brain. Nicotine is still a widely used drug of abuse, and the negative experiences of withdrawal play a major role in perpetuating addiction. As the present research demonstrates, even subtle changes in nicotine exposure, such as brief pre-exposure, can result in drastic changes in anxiety during withdrawal. Understanding

how and when nicotine withdrawal causes anxiety informs better therapies and more effective treatments. Our results indicate that anxiety from nicotine withdrawal is strongest early in withdrawal (Figures 1, 2), however this anxiety is extremely sensitive to the nature of prior nicotine administration (Figures 7, 8, 9). Knowing when the best time to quit is in order to minimize effects of withdrawal could make the difference between success and relapse, and we look forward to future research exploring these questions.

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## Figure Captions

*Figure 1.* Percentage change scores in males on Test 1 from Experiment 1. Brackets represent standard errors.

*Figure 2.* Percentage change scores in females on Test 1 from Experiment 1. Brackets represent standard errors.

*Figure 3.* Percentage change scores in males on Test 2 from Experiment 1. Brackets represent standard errors.

*Figure 4.* Percentage change scores in females on Test 2 from Experiment 1. Brackets represent standard errors.

*Figure 5.* Percentage change scores in males on Test 3 from Experiment 1. Brackets represent standard errors.

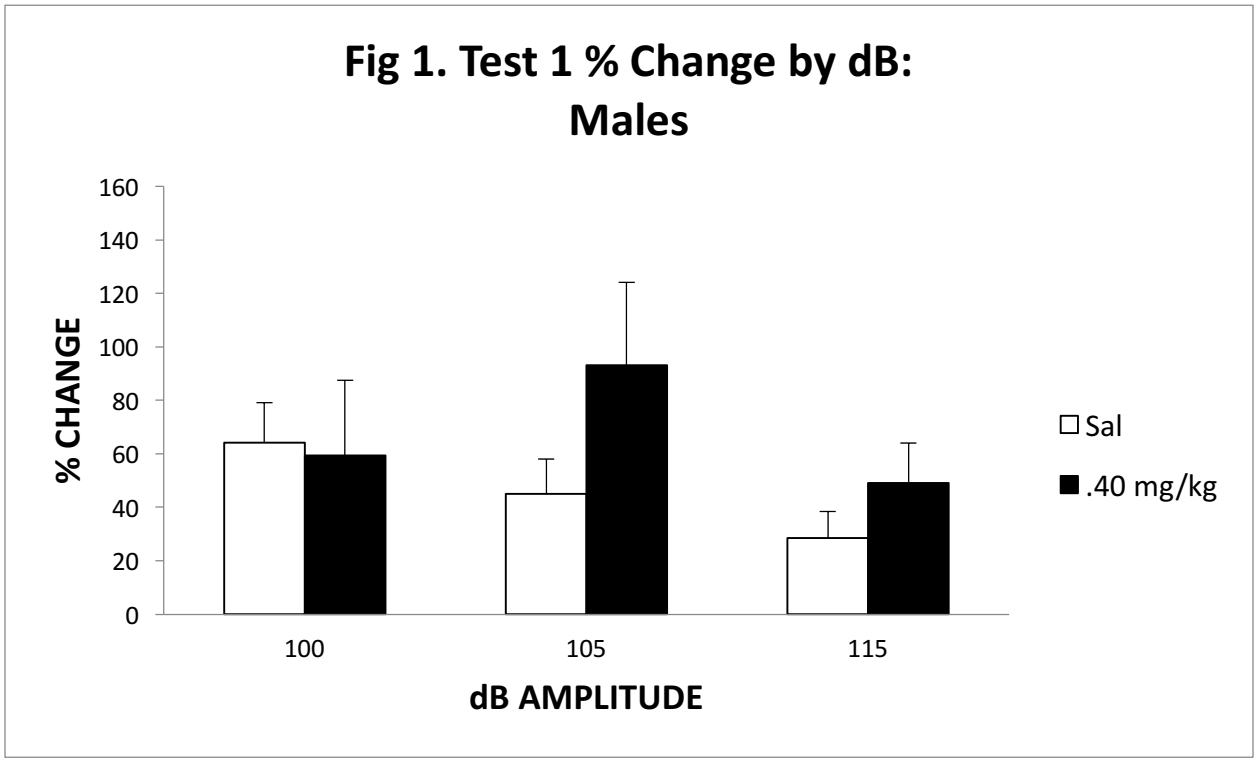
*Figure 6.* Percentage change scores in females on Test 3 from Experiment 1. Brackets represent standard errors.

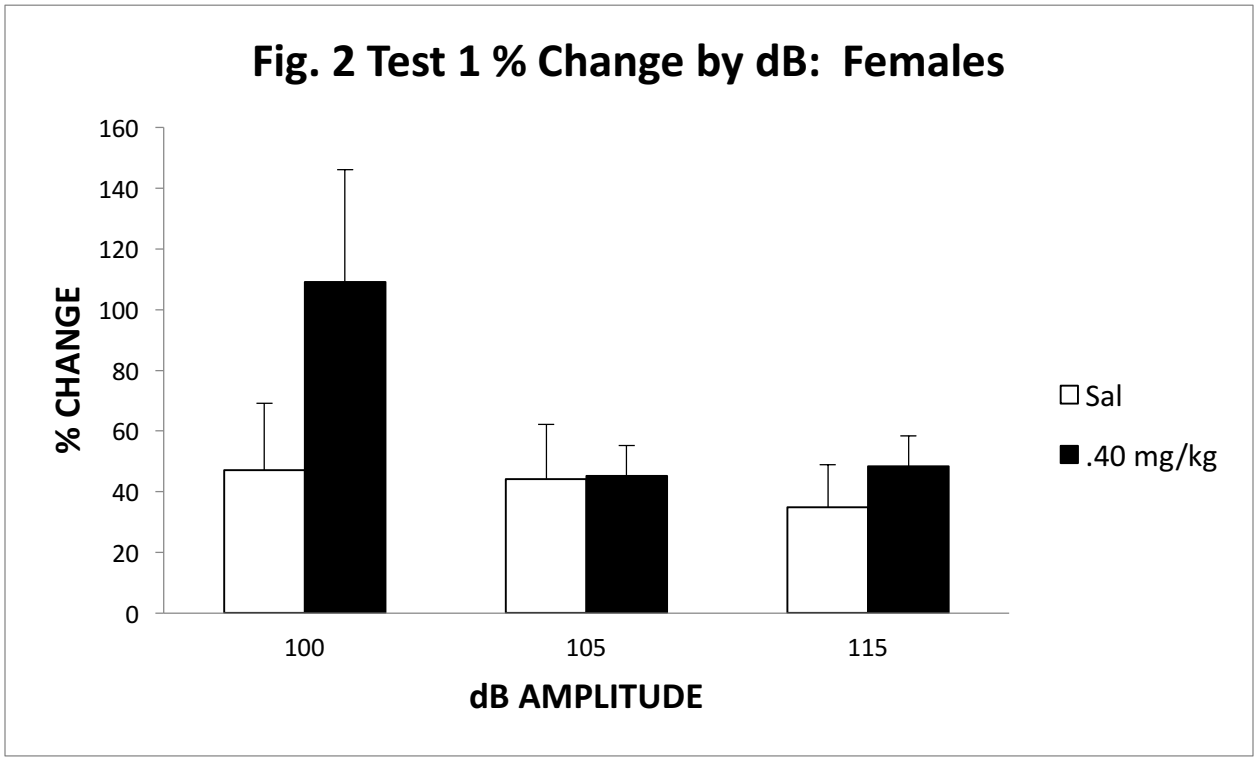
*Figure 7.* Percentage change scores in males and females on Test 1 from Experiment 2. Brackets represent standard errors.

*Figure 8.* Percentage change scores in males and females on Test 2 from Experiment 2. Brackets represent standard errors.

*Figure 9.* Percentage change scores in males and females on Test 3 from Experiment 2. Brackets represent standard errors.

EXPERIMENT 1



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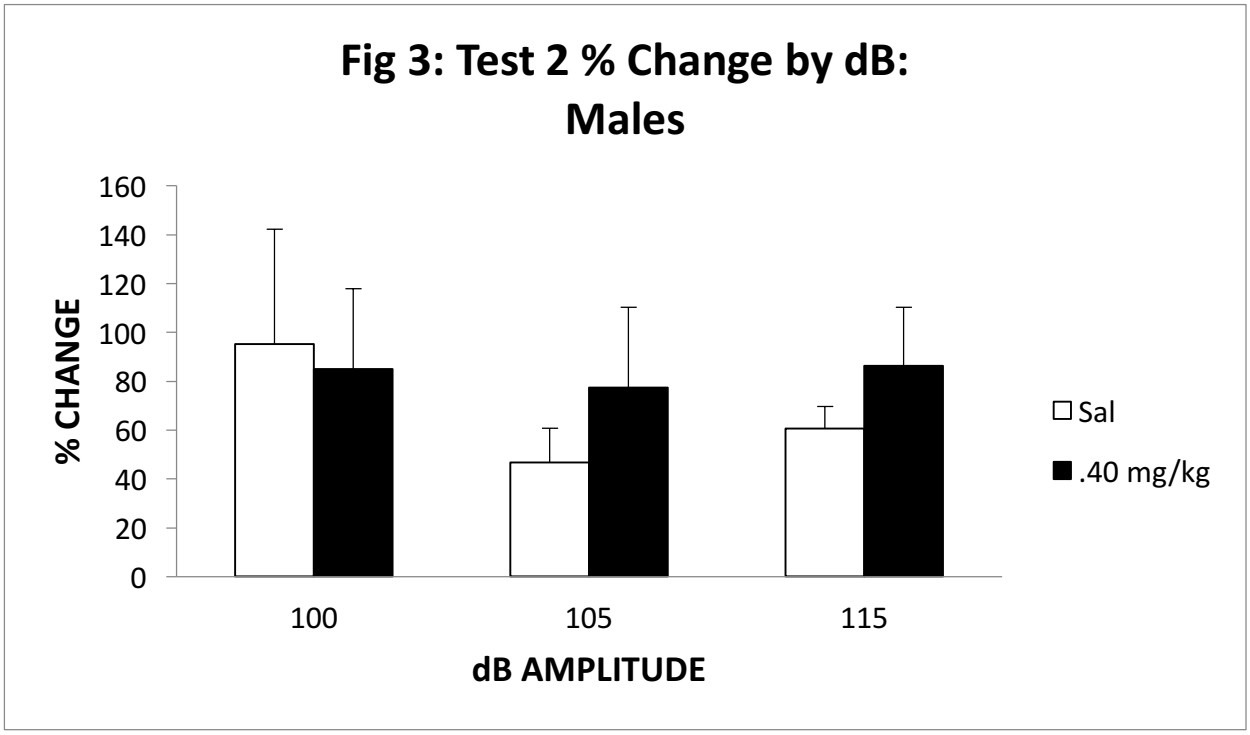


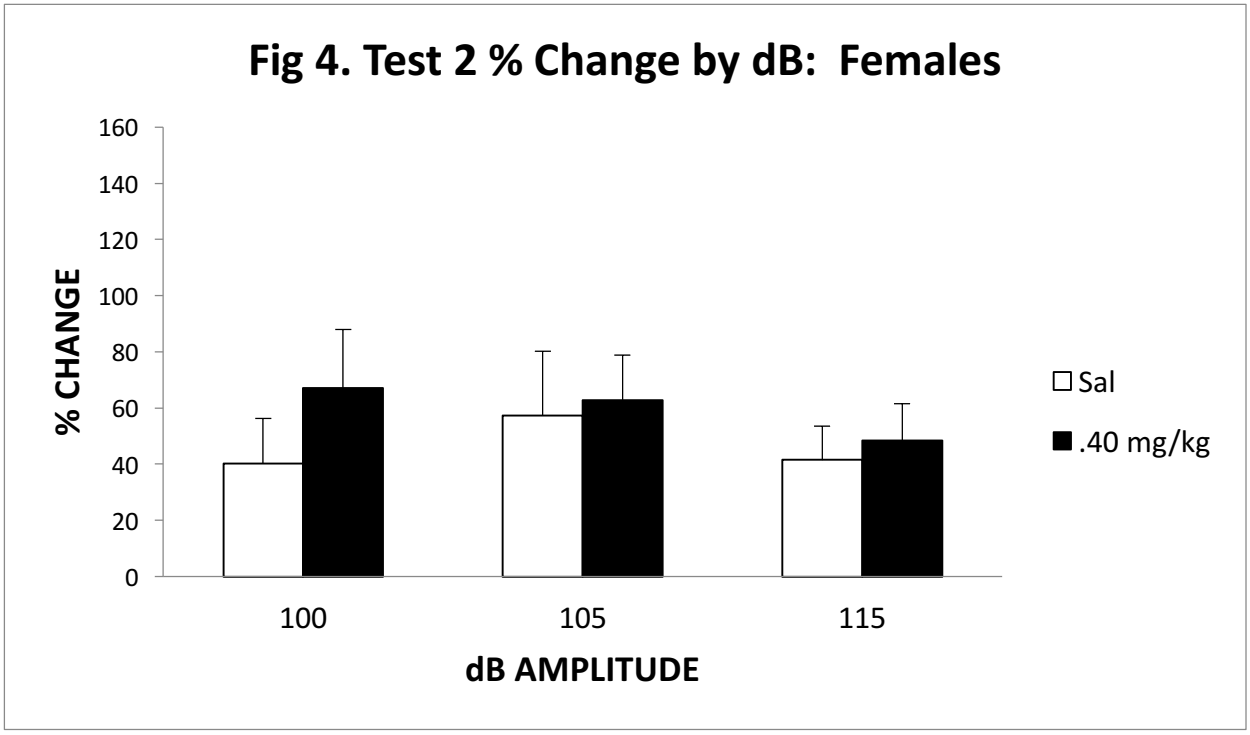

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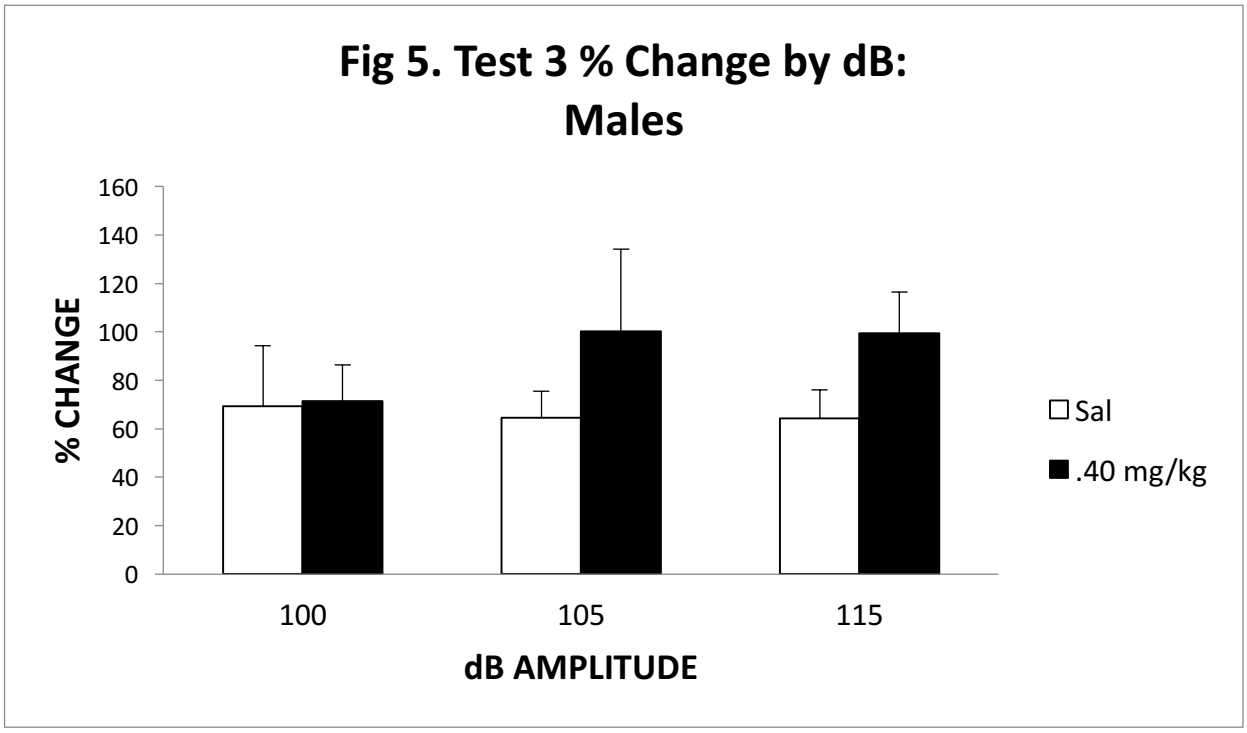
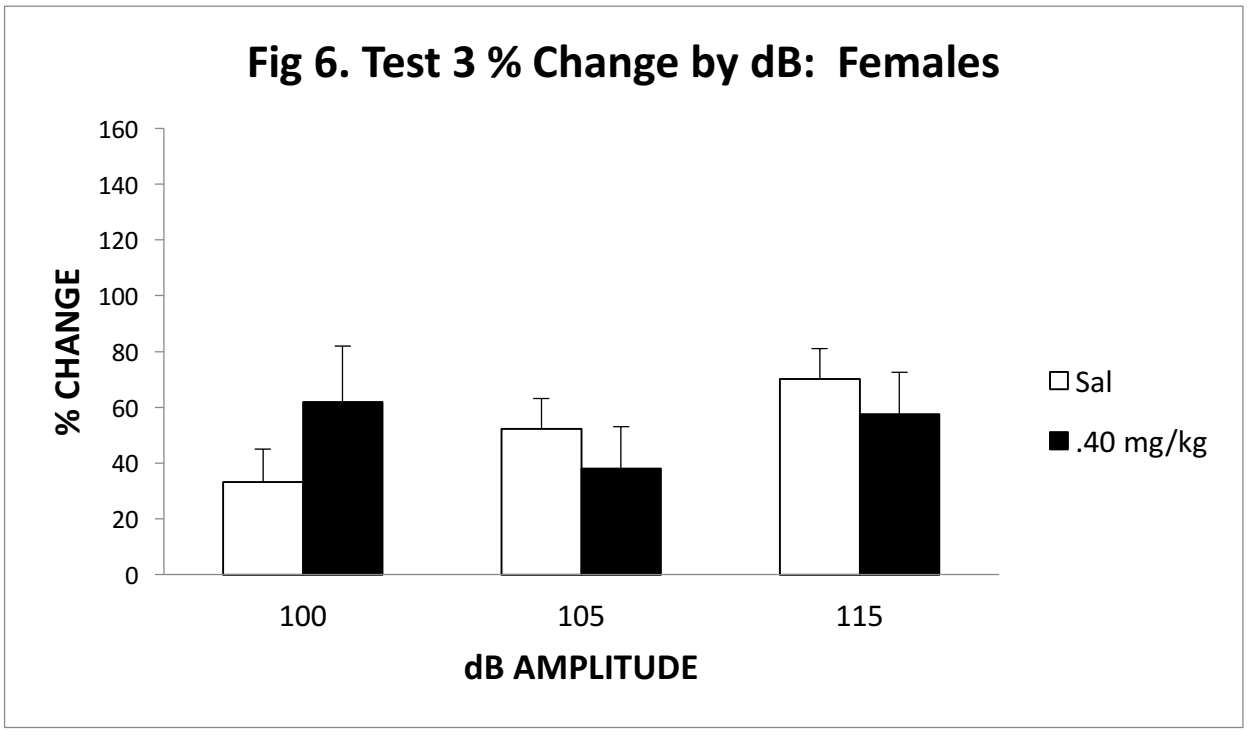
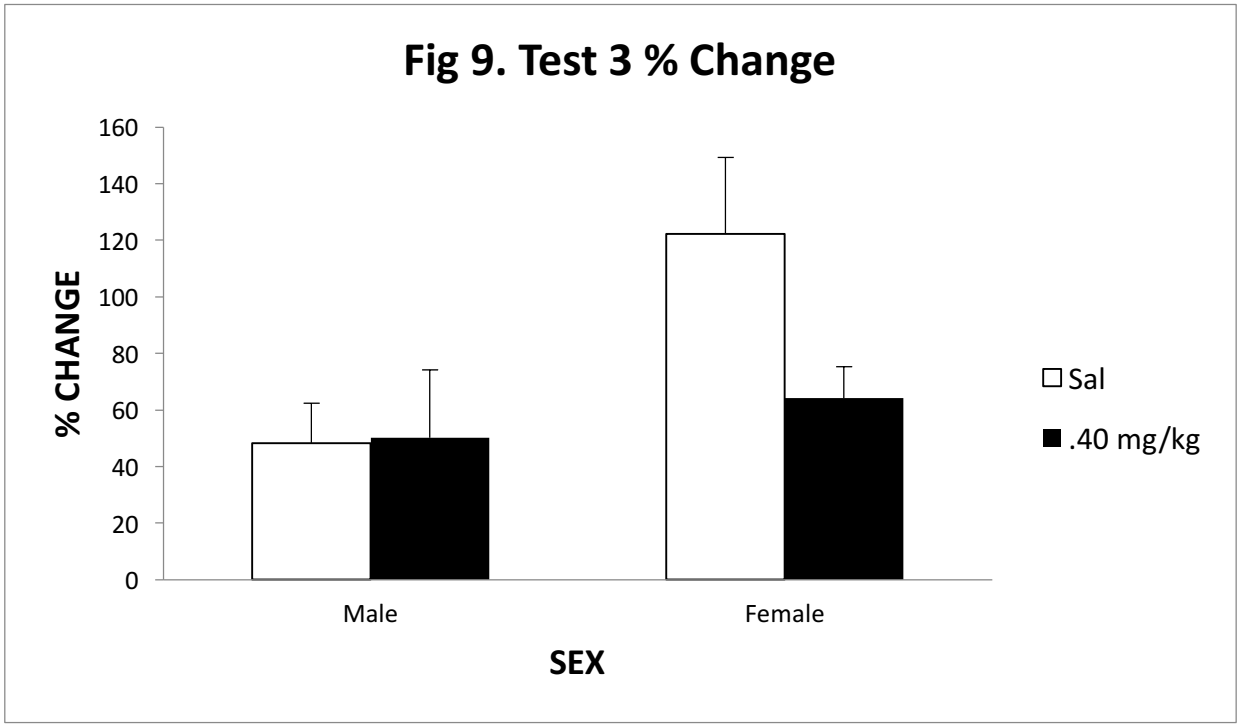


EXPERIMENT 2

EXPERIMENT 2

EXPERIMENT 2



## APPENDIX A: Literature Review

In addition to the focused empirical report of this Thesis above, a broader literature review was conducted in preparation of this Thesis. The literature review is included in what follows. The literature review is presented separately to allow more detailed consideration of topics related to but outside the scope of the focused empirical report that comprises the main Thesis.

## Review: Molecular and Behavioral Effects of Nicotine Administration and Withdrawal

### Introduction

Nicotine is a historically significant drug in our society, and especially for Virginia in the context of tobacco cultivation. Current scientific understanding of how nicotine interacts with the brain is comprehensive, however more research is needed to explore how nicotine produces addiction and how the negative symptoms of withdrawal manifest in the brain. The goal of this review was to provide a comprehensive overview of nicotine as a psychoactive drug, and to provide focus to molecular and behavioral aspects of nicotine withdrawal that contribute to continued nicotine addiction.

Cigarette smoking and tobacco usage in the United States is on the decline, yet still 17% of adults smoke cigarettes (CDC 'Current', 2015). Cigarette smoking and nicotine use is extremely addictive, in a given one-year period over 50% of smokers will attempt to quit, while only 6% will actually be able to stop smoking (CDC 'Prevalence', 2015). Alternative tobacco products such as e-cigarettes have also rocketed in popularity in the past decade, and up to 20% of individuals who quit cigarettes in a given year continue to use electronic cigarettes containing nicotine (CDC 'Electronic', 2015). The adverse effects of nicotine withdrawal such as depression, physical pain, and increased anxiety are factors that promote relapse and prevent successful attempts to quit (Vorvick, 2013). Understanding the negative effects of withdrawal and when these effects are strongest is critical to enhancing the effectiveness of intervention efforts, and to progressing our knowledge about addiction by extending analysis of the effects of addiction beyond the period of drug exposure. Continued study of nicotine on the molecular, systems, and behavioral level is necessary as long as nicotine is a common drug of abuse.

**Nicotine Chemistry and Metabolism**

Nicotine is a psychoactive alkaloid compound that is naturally produced in many common plants (Yildiz, 2004). Historically, nicotine is most readily associated with the tobacco plant and is found in tobacco products sold today such as cigarettes, cigars, chewing tobacco, and e-cigarettes. Evolutionarily, nicotine is thought to function as a natural pesticide, and is found in trace amounts in many cultivated foods (Domino et al., 1993). The highest nicotine concentration (ng/g) of any plant found in nature is actually eggplant, followed by tomatoes. (Domino et al., 1993). Because of its prevalence, many non-smokers have trace amounts of nicotine in their blood, although the amount pales in comparison to that found in people who use nicotine as a recreational drug (Domino et al., 1993).

Structurally, Nicotine [$C_{10}H_{14}N_2$] is an amine, and is composed of pyrrolidine and pyridine rings and its structure and size allow it to easily cross biological membranes (Yildiz, 2004). Free nicotine is highly basic, and it must be neutralized to within range of physiological pH in order to maximize absorption through the lungs, mouth, mucous membranes, gastrointestinal tract, or skin (Yildiz, 2004). Like all drugs of abuse, nicotine freely and quickly crosses the blood-brain barrier once inside a biological system (Yildiz, 2004). After absorption, nicotine metabolism occurs primarily in the liver, and up to 80% is converted into Cotinine, its primary metabolite, and assays for cotinine are relatively reliable ways to assess nicotine exposure both clinically and experimentally (Yildiz, 2004). The majority of absorbed nicotine is expelled as cotinine in urine, however it can be excreted from the body through any major gland system such as saliva, tears, or sweat (Yildiz, 2004).

Once in the brain, nicotine produces somatic responses by activating the parasympathetic nervous system (Yildiz, 2004). These responses are generally uniform across species and include

an increase in blood pressure and heart rate (Yildiz, 2004). Cigarettes have long been known to lead to lung disease and lung cancer, and the main causes of these disease are the tar and other caustic chemicals found in manufactured tobacco products (Yildiz, 2004). Little less known is the fact that nicotine also contributes to the pathology of these diseases by increasing levels of cellular oxidative stress and increasing the production of free radicals, both of which can lead to serious pathologies (Yildiz, 2004).

**Nicotinic Signaling**

Once in the brain, nicotine binds to nicotinic acetylcholine receptors (nAChRs), which normally bind the endogenous excitatory neurotransmitter acetylcholine (ACh) (Pistillo et al., 2014). Nicotine is an nAChR agonist, and activates the nAChRs when it binds mimicking its action (Pistillo et al., 2014). Structurally, nAChRs are pentameric channel proteins that are usually expressed as a combination of one type of 8 known alpha subunits and one type of 3 known beta subunits (Pistillo et al., 2014). Different subtypes of nAChRs have been shown to mediate different neuronal functions and are expressed in distinct brain regions (Pistillo et al., 2014). Functionally, nAChRs are ionotropic receptors, meaning that once activated they allow sodium and potassium ions to flow across the neuronal membrane down their electrical and chemical gradients (Pistillo et al., 2014). Besides sodium and potassium, calcium has also been shown to interact with nAChRs, which implies that the receptor and its ligands play a role in regulating the meta-plasticity of neural circuits, and this has critical implications for nicotine as an addictive drug (Pistillo et al., 2014). nAChR signaling is intrinsically excitatory, but the net downstream effect of nAChR activation is dependent on the type of neuron that the receptor is found on and which neurotransmitter system is implicated (Pistillo et al., 2014).

The distribution of nAChRs is widespread, and they are found throughout the brain as well as at muscular junctions (Pistillo et al., 2014). One area of particularly high nAChR density and is the mesocorticolimbic system, which contains the brain regions responsible for the maintenance of addiction. The mesocorticolimbic system includes the Ventral Tegmental Area (VTA), the Nucleus Accumbens (NAcc) and the ventral striatum, as well as the Pre-Frontal Cortex (PFC) and all of the connecting neural tracts (Pistillo et al. 2014). Besides the being the neural circuits responsible for drug addiction, these regions also mediate processes such as motivation, decision making, and perceived value (Pistillo et al., 2014). Nicotine withdrawal has been shown to reduce brain reward function, just one behavior of many that is critically dependent on these brain regions (Epping-Jordan et al., 1998).

In the brain, addiction is characterized by a drastic increase in dopaminergic signaling resulting in a behavioral shift towards drug seeking behavior (Paolini & De Biasi, 2011). Like many other drugs of abuse, exposure to nicotine changes DA utilization in the VTA, PFC, NAcc, and dorsolateral striatum (George et. al 1998, Paolini & De Biasi 2011). Nicotine also triggers neurotransmitter release in the hippocampus and amygdala, brain regions which contain the neural circuits that take part in the processes of learning and memory and contribute to the overall emotional state of individuals (Fu et al., 1998). Fu et al. (1998) demonstrated that when nAChRs in the hippocampus and amygdala are activated by nicotine the excitatory neurotransmitter norepinephrine (NE) is released, implying that nicotine can molecularly alter how individuals learn and store memories (Fu et al., 1998). Nicotinic signaling in the brain has a wide range of both somatic and molecular effects, and the changes produced by nAChR activation can have direct implications for behavior.

**Mechanics of nAChRs in Nicotine Addiction**

Addiction to nicotine has a molecular basis, and in the brain addiction is characterized by a net decrease in excitatory nAChR signaling (Pistillo et al., 2014). Chronic nicotine administration causes an upregulation in the levels of nAChRs, and even though nAChR signaling is excitatory, the increase in the number of nAChRs following nicotine administration leads to a decrease in endogenous ACh signaling. When the brain is exposed to nicotine and after the upregulation of nAChRs, the amount of biologically produced ACh is no longer sufficient to activate nAChRs at the same level as before nicotine exposure (Quick & Lester, 2002). In individuals that are repeatedly exposed to high doses of nicotine, such as chronic smokers, the brain responds to the near continuous activation of nAChRs by increasing the density of these receptors on neuronal membranes (Quick & Lester, 2002). This up-regulation the in amount of receptors causes a depression in nAChR signaling, which can also be described colloquially nAChRs having a "thirst" for ACh (Quick & Lester, 2002).

When nAChRs are repeatedly activated by endogenous ACh or an agonist such as nicotine, they become desensitized, meaning that the magnitude of response to a specific amount of activation decreases following each subsequent activation (Giniatullin et al., 2005). This desensitization is therefore a use dependent manner in which certain brain areas are chronically depressed after high levels of nAChR activation (Giniatullin et al., 2005). Nicotine has been shown to differentially desensitize nAChRs compared to endogenous ACh (Giniatullin et al., 2005). Specifically, certain subtypes of ACh take a longer time to re-sensitize after exposure to nicotine compared to other agonists, especially subtypes containing the $\alpha_7$ subunit (Giniatullin et al., 2005). Different nAChR subtypes vary in the speed and duration of desensitization, and these properties have been shown to have direct correlates to the molecular structure of the receptors

(Giniatullin et al., 2005). Nicotine thus interacts with nAChRs to produce significant molecular and system level changes that lead to addiction and dependence.

**NAChRs Mediate Anxiety of Nicotine Withdrawal**

Several different specific subtypes of nAChRs are implicated as being crucial mediators of both general anxiety and anxiety produced by addiction and withdrawal. Roni & Rahman (2001) demonstrated that administering lobeline, a specific $\alpha_4\beta_2$ subtype nAChR antagonist, significantly reduced the anxiety in awake behaving animals as measured in EPM. However, research into the role that the $\beta_2$ subunit plays in mitigating the anxiety of withdrawal is mixed. Caldarone et al. (2008) found that $\beta_2$ knockout mice exhibited no significant differences in anxiety determined by EPM performance. It should be noted that the discrepancy between the Roni & Rahman (2011) and Caldarone et al. (2008) studies could be due to differences in the animal models used, i.e. rats vs. mice.

Additionally, Meyers et al. (2015) have shown that subtypes of nAChRs containing a $\beta_4$ subunit are critically important in the molecular process of the anxiety of both chronic and acute nicotine administration. Genetically deleting the $\beta_4$ subunit resulted in an almost complete absence of the somatic signs of nicotine withdrawal in an animal model (Meyers et al., 2015). $\beta_4$ subunits are highly expressed in the Medial Habenula, and Frahm et al. (2011) demonstrated that the Medial Habenula is a crucial component in the neural circuitry responsible for nicotine consumption, and furthermore that the region is sensitive to the ratio of different nAChR subtypes present. Together these results demonstrate the role that various nAChR subtypes play in the physical process of withdrawal, and elucidate possible molecular targets for the treatment of the anxiety and stress caused by nicotine withdrawal.

**Neural Changes Resulting from Nicotine Administration and Withdrawal**

AChRs are extremely sensitive to even a small amount of nicotine, and discrete doses can significantly alter neurotransmitter systems across the brain. George et al. (1998) have shown significant dose differential changes in DA utilization occur in the NAcc, medial pre-frontal cortex (mPFC), and the dorsolateral striatum, following just 4 days of nicotine administration. Adult rats were given once daily injections of saline, .15 mg/kg or .60 mg/kg nicotine for 4 days followed by a drug challenge and behavioral stressor (foot shock) on day 5 (George et al., 1998). The low .15 mg/kg dose of nicotine coupled with the stress of foot shock significantly increased DA utilization in the mPFC and NAcc, however the high .60 mg/kg dose coupled with stress only increased DA utilization in the dorsolateral striatum (George et al., 1998). These findings demonstrate that nicotine has diverse dose dependent effects on neurotransmitter systems, and that these effects are inexorably tied to biological stress.

Arnold et al. (2003) have demonstrated a similar effect of brief nicotine exposure having drastic impacts on neurotransmitter systems. Following 4 days of pretreatment with either vehicle or .4 mg/kg I.P. nicotine injections, a subsequent nicotine injection on day 5 produced significantly higher cortical levels of ACh in animals previously sensitized to nicotine (Arnold et al., 2003). The same significant increase was found in animals challenged on day 8 following sensitization, indicating a lingering effect of sensitization (Arnold et al., 2003).

Additionally, the physiological and morphological effects produced by nicotine administration can be long-lasting. Brown & Kolb (2001) have shown that significant brain changes occur following moderate nicotine exposure and persist even after 2 weeks of abstinence. Specifically, animals exposed to nicotine had significantly increased dendrite length and dendritic spine density in the NAcc, a region crucial in the process of addiction. Brown &

Kolb (2001) also demonstrated that there was some degree of behavioral sensitization to an acute dose of nicotine following 2 weeks of abstinence, meaning that the long lasting morphological changes are directly related to behavioral changes. Interestingly, Brown & Kolb (2001) used a drug administration schedule that is considered light, in which rats were given a sub cutaneous injection of .7 mg/kg nicotine 3 times a week for 5 weeks, followed by 2 weeks of abstinence. Together, these papers illustrate how even small doses of nicotine can produce both immediate, and long lasting neural changes that can translate to behavioral changes in behaving animals

**Animal Models of Anxiety**

Animal models of anxiety can be used to study how withdrawal from nicotine can influence stress and anxiety in individuals, and how these events can contribute to addiction. One of the most useful behavioral assays for measuring anxiety in animals is a procedure called Light-Enhanced Startle (LES). The LES paradigm is a validated animal model of anxiety, and has been used to examine the neuroanatomical and pharmacological basis of anxiety before (Walker & Davis, 1997). In LES, rats are exposed, either in the presence of a bright light or darkness, to brief bursts of white noise that elicit an acoustic startle reflex (Walker & Davis, 1997). Rodents, normally nocturnal, naturally find bright light anxiety producing (Walker & Davis, 1997). This means that the magnitude of the elicited startle reflex is larger in the presence of a bright light as compared to darkness (Walker & Davis, 1997). A key component of the LES paradigm is that it models un-conditioned anxiety, as opposed to fear learning (Walker & Davis, 1997). This means that the startle response does not habituate, and that the same subjects can be repeatedly tested over time (Walker & Davis, 1997). In addition, LES has shown to be susceptible to drug manipulation, both anxiogenic and anxiolytic, making it ideal for investigation of nicotine (Walker & Davis, 1997).

Another salient assay of behavioral anxiety is the elevated plus maze (EPM). The EPM is used as an assay for behavioral anxiety in rodent models, in which animals traverse "open" versus "closed" arms of a simple plus maze. Anxiety is determined as a function of how much time the animal spends in the open arms or the number of times it enters an open arm, indicating a less anxious state. An additional common assay for behavioral anxiety is the open field test, in which rodents are placed in an enclosed area. Anxious behavior is defined as more time spent against walls or in corners as compared the open area. Both of these tests rely on the fact that rodents are nocturnal, and bright and open spaces are known to be naturally aversive to them. The social interaction test is yet another viable assay for behavioral anxiety, in which animals are introduced to a common area and their interactions with other animals are observed. In this model anxiety behavior is described as more time spent alone or being "anti-social". This assay is useful for determining how anxiety may be interacting with other complex behaviors in awake animals. These different experimental methods represent the unique ways in which researchers can approach the anxiety produced by nicotine withdrawal using animal models.

**Anxiety of Nicotine Withdrawal and Startle Paradigms**

Spontaneous withdrawal is the natural "wearing off" of a drug as it is metabolized in the body and its effect diminished, and is the type of withdrawal experienced by smokers. During nicotine withdrawal, DA signaling is increased in the mesolimbic system as well as the NAcc, and this alteration in the NAcc is primary cause of withdrawal symptoms (Paolini & De Biasi, 2011). The Bed Nucleus of the Stria Terminalis (BNST) is also critically implicated as the brain region responsible for modulating innate anxiety (Davis et al., 2010). Additionally, the BNST, outer shell of the NAcc, and the Central Nucleus of the Amygdala (CeA) together make up a region known as the extended amygdala, a superstructure of interconnected brain regions

responsible for mediating anxiety behaviors in the brain that is incredibly sensitive to nicotine withdrawal (Marcinkeiwcz et al., 2009). Marcinkeiwcz et al. (2009) demonstrated that nicotine withdrawal in the extended amygdala is associated with a significant increase in tissue levels of the stress-marker Corticotrophin Releasing Factor (CRF), and that this increase is responsible for the negative affective behaviors associated with nicotine withdrawal. Torres et al. (2013) further confirms that spontaneous nicotine withdrawal increases CRF in the brain regions of the extended amygdala such as the NAcc.

In the only currently published paper examining the effect of nicotine withdrawal in LES, Jonkman et al. (2008) exposed rats to nicotine using an osmotic minipump then 24 hr after the nicotine dosing period ended (during acute withdrawal) tested the animals in LES and observed that nicotine treated rats showed greater LES than control rats. Jonkman et al. (2008) administered nicotine via osmotic minipumps which dose nicotine continuously over each 24 hr period while the pumps are active (not unlike a nicotine patch). Using Potentiated Acoustic Startle Response, a behavioral assay very similar to LES but foregoing light as an environmental stressor, and intraperitoneal (IP) injections for drug delivery Engelmann et al. (2009) further confirm that withdrawal from nicotine modulates anxiety and can be measured as a response to a startle stimulus. Compared to continuous subcutaneous delivery, IP injections of nicotine are thought to more accurately model the rapid rises in nicotine brain and blood levels associated with cigarette smoking, as well as the period of abstinence that occurs between cigarettes. Repeated dosing and subsequent spontaneous withdrawal from discrete doses of nicotine produces an increasingly anxiogenic effect in male rats in a positive dose dependent manner (Engelmann et al., 2009). A second dose of nicotine during testing delayed the potentiated startle effect, implying that nicotine can be both anxiogenic or anxiolytic depending on the state of

addiction and withdrawal (Engelmann et al., 2009). In comparison to Jonkman et al. (2008), animals exhibited elevated baseline responding, meaning that while withdrawal from chronic nicotine may not affect baseline levels of anxiety, withdrawal from discrete doses may result in a generally higher anxious state (Engelmann et al., 2009). These results provide significant evidence for the validity of using startle paradigms to evaluate the anxiety of nicotine withdrawal.

**Anxiety of Nicotine Withdrawal Varies by Age and Sex**

The effects of nicotine on the brain are sensitive to age, and understanding how nicotine works differently in the developing brain is important considering that nicotine is often used by adolescents. Research on anxiety of nicotine withdrawal in adolescents is conflicted. Torres et al. (2013) found that the effects of nicotine withdrawal are less pronounced in adolescent rats as compared to adult rats as measured by EPM. Shram et. al (2008) however, demonstrated that there is a non-significant difference in the Conditioned Place Aversion experienced by adolescents and adults during spontaneous nicotine withdrawal following a dosing schedule similar to Torres et. al (2013). Cheeta et al. (2001) found that nicotine administration in adolescents is actually anxiolytic in the context of the social interaction test. Any age related difference is most likely due to differences in metabolism and neural plasticity, and more research needs to be conducted on the effects of adolescent exposure.

Withdrawal from chronic nicotine appears to affect males and females differently. Torres et. al (2013) found that female rats treated with chronic nicotine exhibit significantly increased anxiety during spontaneous withdrawal in both EPM and the open-field compared to males. Females also expressed significantly higher levels of CRF mRNA and blood levels of corticosterone in the NAcc, which are valid indicators of biological stress in a system (Torres et

al., 2013). These findings were also echoed in a paper by Caldarone et al., (2008) which looked at anxiety in male and female mice following chronic nicotine administration. In this particular study researchers found that anxiogenic behavior in EPM following chronic nicotine was only present in female mice (Caldarone et al., 2008). Additionally, Caldarone et al. (2008) demonstrated that female mice exhibited a decrease in locomotor activity corresponding to the psychostimulant aspects of nicotine administration. In their study, Cheeta et al. (2001) found the anxiolytic effect seen in nicotine treated adolescents in the social interaction test was much higher in females as compared to males, providing another example of how sex differences in brain manifest as differential responses to nicotine.

**Conclusion**

Nicotine is a widely used recreational drug, and it is extremely addictive. In the brain, nicotine activates nAChRs, resulting in excitatory activation of many different neurotransmitter systems. Nicotinic signaling has a wide range of effects and can influence processes such as stress, anxiety, emotion, learning, memory, and addiction depending on the brain regions activated. Even light nicotine administration can cause a diverse range of neural changes, and these changes can be both fast acting and long lasting. The structures of the extended amygdala including the amygdala, NAcc, and BNST are critical components of the brain's anxiety pathway, and these structures are highly susceptible to manipulation by nicotine. Nicotine addiction is marked by a desensitization and up-regulation of nAChRs in these brain structures, and this leads to an increase in excitatory signaling and activation in the absence of nicotine. Specific nAChR subunits have been directly linked to the aversive aspects of nicotine withdrawal, and these negative symptoms are a major contributor to nicotine's addictive properties. Animal models of anxiety provide salient tools for studying the anxiety of nicotine

withdrawal, and in particular LES is an effective tool for determining the effects neural changes produced by nicotine have on behavior. Literature suggests that withdrawal from nicotine is generally anxiogenic, but this can depend on both sex and age. In general, adolescents seem to be less susceptible to the anxiogenic effects of nicotine withdrawal. Additionally, females tend to exhibit increased anxiety as compared to males during spontaneous withdrawal. Overall, nicotine and its effects on the brain are important research topics, and the behavioral and molecular study of nicotine withdrawal deserves continued research.

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