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The Role of Time and Nicotine Dose on Anxiety Measured with Light Enhanced Startle

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Abstract

Two experiments using Light-enhanced startle (LES) examined dose-dependent and time-dependent effects of acute nicotine on anxiety. In Experiment 1 rats were exposed to saline, .15 mg/kg, or .40 mg/kg (i.p.) nicotine and 5 minutes later were behaviorally tested in LES. Data suggested that nicotine at both doses was anxiolytic in males but not anxiolytic in females. In females, the higher nicotine dose, .40 mg/kg, was anxiogenic but only during later portions of the test session. In both males and females, within-session variation in LES provided evidence that LES increased in magnitude as time since nicotine administration increased. Therefore, in Experiment 2, longer drug-to-test intervals were applied in order to examine possible time-dependent increases in anxiety produced by nicotine. In Experiment 2, rats were exposed to saline or .40 mg/kg nicotine and were behaviorally tested 15 min or 35 min after nicotine administration in LES. Trends in the available data suggested an anxiogenic profile of nicotine when tested 15 min following drug administration but an anxiolytic profile when tested 35 min following drug administration. At the short 5 min drug-to-test interval used in Experiment 1, findings contradict those in other experiments using the social interaction test of anxiety. Collectively, results suggest Light-enhanced startle is sensitive to dose and time-dependent effects of nicotine on anxiety. Possible differences between reflexive and non-reflexive measures of fear and gonadal influences in anxiety expression are discussed.

The Role of Time and Nicotine Dose on Anxiety Measured in Light Enhanced Startle

A recent article published by the American Heart Association indicates that Americans are more likely to use electronic cigarettes than traditional cigarettes because Americans believe that electronic cigarettes are less harmful (American Heart Association, 2019). Though under current debate, even if it is found to be true that e-cigarette vapor contains fewer contaminants than traditional tobacco smoke, the primary neuroactive compound in these devices, nicotine, is still present and can alter typical brain function (American Heart Association, 2019; World Health Organization, 2019). Although nicotine has been studied extensively in the past 40 years the effects of this compound in the brain, which are known to produce anxiety behavior in both humans and animal models, remains unclear (Parrot, 1999).

Nicotine is a nicotinic acetylcholine receptor (nAChR) agonist and parasympathetic alkaloid with a high affinity for the $\alpha 2\beta 4$ nAChR subtype (Abou-Donia, 2015). Stimulation of nAChRs by nicotine has been shown to modulate anxiety in stressful situations (Piccioto et al., 2002; Irvine, Cheeta, & File, 2001). Molecularly, nicotine alters the central nervous system by binding to nAChRs which then causes this ligand gated ion channel to turn and open (Wonnacott et al., 2005). The activation of these nAChRs causes an influx of calcium and sodium ions because of said consequential opening of the channel (Kirsch et al., 2016). This molecular cascade can generate or dissipate states of anxiety through its interactions in the basal lateral amygdala (BLA), an area of the brain which is widely recognized for its involvement and production of neural responses to anxiety inducing stimuli (Sharp, 2019). Activation of glutamatergic output neurons of the BLA in part characterize the brain's response to stress-inducing environmental stimuli and play a central role in anxiety. Clinically aberrant activity of the BLA, for example, has been shown to be associated with exaggerated stress responses leading to the progression of anxiety disorders in humans (Graham & Milad, 2011). Importantly, both glutamatergic and GABAergic neurons of the BLA express nAChRs. Stimulation of nAChRs located on glutamatergic neurons of the BLA can directly increase excitatory output of the BLA. Moreover, previous research has shown that increases in glutamatergic responses via nAChR stimulation can contribute to changes in anxiety-like behavior in rats (Ryu et al., 2017). However, nAChRs also expressed on GABAergic afferents to the BLA can increase GABA transmission and modify neural activity by decreasing the glutamatergic excitatory output of the BLA. Through this mechanism, nicotine could potentially decrease anxiety through increased transmission of inhibitory GABA neurons. In summary, both excitatory and inhibitory modulation of the BLA can be mediated by nicotine stimulating nAChRs within the circuitry of the BLA. This is important to nicotine's relationship with anxiety because the glutamatergic neurons expressing nAChRs and GABAergic neurons expressing nAChRs reveal different routes of activation on BLA that could either increase or decrease anxiety.

Animal models are especially valuable in isolating causal influences of nicotine on anxiety. File, Gonzalez, & Andrews (1998) for example, demonstrated in rats that decreases in anxiety measured in the social interaction test, possibly due to nicotine, were blocked by the nonselective nAChR antagonist mecamylamine; revealing a causal role of nAChRs in anxiety. In the social interaction test, anxiety is measured as a reduction in normal prosocial behavior. However, consistent with the dual routes through which nicotine can affect BLA activity described above, there is a current debate in the literature whether nicotine is anxiolytic or anxiogenic in the social interaction test among others. Some studies report nicotine is anxiolytic (Brioni et al., 1993; Costall et al. ,1989; Vale & Green, 1986; Villégier, et al., 2010) whereas others report that nicotine is anxiogenic (Ouagazzal, Kenny, & File, 1999a, 1999b; Morrison, 1969). These paradoxical results could be attributed to the behavioral paradigm used, differences

in dose and route of administration of nicotine, and the nicotine-to-test interval used, among other experimental parameters (for a review see Picciotto, Brunzell, & Caldarone, 2002; see Morissette et al., 2007 for a related discussion of interrelationships between nicotine and anxiety in humans).

Assessment of the delay between nicotine administration and behavioral (or neural) testing is a critical factor because nicotine can modulate the release of neurotransmitters in different parts of the brain and it is likely that nAChRs have different time courses of inactivation and activation which could have time-dependent effects on brain systems important to anxiety (Picciotto et al., 2002). Interestingly, some studies using the *same* behavioral task report differing effects of nicotine when the nicotine-to-test interval is varied. In one striking example, Irvine, Cheeta, and File (1999) varied the nicotine-to-test delay and reported in the social interaction test with rats that the same dose of nicotine (.1 mg/kg) had anxiogenic effects with a 5 min nicotine-to-test interval, but anxiolytic effects at a longer 30 min nicotine-to-test interval. Thus, the same dose of nicotine had opposite effects on behavior. The current research systematically examines the impact of a nicotine-to-test interval similar to that of Irvine et al. (1999) but examines anxiety in light-enhanced startle, a validated animal model of anxiety (Walker & Davis, 1997). Unlike Irvine et al. (1999) the current research additionally examines the effect of nicotine dose on anxiety, and explores possible sex differences.

In the light-enhanced startle (LES) paradigm the rat's acoustic startle reflex to brief but loud bursts of white noise is measured in a dark setting and then is measured again in the presence of a bright light, an innately aversive stimulus to rodents (Walker & Davis, 1997). In this model, high illumination levels are thought to increase anxiety and thereby increase the magnitude of the acoustic startle reflex in light compared to dark sessions (Walker & Davis 1997). This paradigm advantageously provides a way to assess acoustic startle response to unconditioned fear stimuli implicated in anxiety (de Jongh et al, 2003). Additionally, research has shown that increases of startle reflex in LES reflect an influence of higher anxiety levels while low levels of startle reflect states of lower anxiety (Walker & Davis 2001). LES also provides further advantages over a related paradigm, fear-potentiated startle (FPS), another model of anxiety, because LES reflects unlearned fear to unconditioned stimuli whereas FPS is specifically designed to assess anxiety responses to learned, conditioned fear stimuli. As a result, LES can be repeatedly tested without contaminating factors of learned fear extinction or conditioned sensitization (Walker & Davis 1997; 2001). Finally, LES serves a valuable tool because it measures an unbiased and 'pure' form of anxiety. As described, LES is not subject to extinction in repeated testing thereby allowing the behavioral measure of anxiety in LES to be uncoupled from learning related changes in anxiety behavior. Additionally, LES, as a startle paradigm using the acoustic startle reflex as primary response measure, engages relatively simple hindbrain circuits (Walker & Davis 1997; 2001) unlike anxiety measured in the social interaction test of Irvine et al. (1999) which is known to recruit higher brain processing centers including the hippocampus (File, Kenny, & Ouagazzal, 1998) and frontal cortex (Ko, 2017). Tests of anxiety in the social interaction test involve choice and decision making whereas anxiety measured in LES involves innate unlearned reflexes. Examining nicotine's effect on anxiety specifically in LES may therefore be of further interest because it will permit comparison of nicotine's impact on reflexive and non-reflexive aspects of fear.

The present research had two goals. Experiment 1 examined the effect of nicotine dose on the magnitude of LES in order to clarify anxiolytic vs. anxiogenic profile of nicotine on anxiety in a startle paradigm as nicotine dose varied, when the nicotine-to-test interval was relatively short. Experiment 2 systematically varied the interval between nicotine administration

and behavioral testing with a single dose of nicotine, similar to that of Irvine et al. (1999), in order to explore time-dependent effects of nicotine on anxiety.

Experiment 1

The goal of Experiment 1 was to examine the relationship between dose of nicotine and anxiety measured in the LES paradigm at a short, 5 min, nicotine-to-test interval. Nicotine's effect on LES has not been systematically examined and there is limited available literature to guide nicotine dose selection in this paradigm. In the social interaction test and elevated plus maze task literature, acute nicotine doses generally vary between 0.1-.0.5 mg/kg, with more limited instances of especially low (e.g. .01 mg/kg) and especially high (e.g., 1.0 mg/kg) doses (e.g., Biala & Budzynska, 2005; File et al., 1998). Nicotine doses of the present experiment will be .15 mg/kg (low dose) and .40 mg/kg (high dose) with a saline group as the control. The low dose condition of Experiment 1 approximates the .1 mg/kg does used by Irvine et al. (1999) who used the social interaction test and who also employed a 5 min nicotine-to-test interval. Additionally, and unlike Irvine et al. (1999) who used only male rats, both male and female rats will be used in the present experiment because some research has shown there to be a difference in the way male and female rats metabolize and react to nicotine (Booze et al, 1999). In Experiment 1, rats were exposed to nicotine (.15 mg/kg or .40 mg/kg, i.p.) or saline, followed by a 5 min delay period and then behavioral testing for LES anxiety.

Method

Subjects

Seventy-two Sprague-Dawley rats (36 male and 36 female) approximately 90 days old were obtained from the breeding colony of the Department of Psychology at The College of William & Mary. Rats were maintained on a 14 hr/10 hr light-dark cycle and were given access to water and food *ad libitum.* All animal care and experimental procedures were carried out in accordance with approved IACUC protocols.

Apparatus

Light-enhanced startle testing occurred in two 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 26-W General Electric compact fluorescent light bulb could be turned on to provide bright illumination of the chamber (1876 lux measured from center of animal 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.

Procedure

Nicotine. Nicotine bitartrate dihydrate salt (MP Pharmaceuticals) was dissolved in saline to create two nicotine dosages: 0.15 mg/ml and 0.40 mg/ml nicotine (free base) and titrated to a pH of approximately 7.4. Nicotine when administered was injected intraperitoneally in a volume of 1 ml/kg body weight creating 0.15 mg/kg and 0.40 mg/kg injection doses.

LES Test Procedure. A baseline drug-free LES test day preceded three nicotine challenge tests in which animals were exposed to saline, 0.15 mg/kg, or 0.40 mg/kg nicotine via intraperitoneal injection in counterbalanced order. 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]). 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 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 (26-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 difference score computed as Ph2 (Light) startle mean – Phase 1 (Dark) startle mean.

*Baseline LES and Nicotine Challenge LES Test Procedure*s. Baseline LES scores were recorded during a preliminary drug-free LES test and used to assign animals into different counterbalanced orders of subsequent nicotine test conditions within which sex and weight were additionally counterbalanced. During nicotine challenge test days animals were exposed to saline, 0.15 mg/kg, or 0.40 mg/kg nicotine administered by intraperitoneal injection. Across the three days of nicotine LES testing each animal received each drug treatment (saline, 0.15 mg/kg, or 0.40 mg/kg nicotine) in a counterbalanced order with each LES test separated by 48 hours. During each test, nicotine was administered at the beginning of the 5 minute wait interval that separated Phase 1 (dark) and Phase 2 (light).

Results and Discussion

Data from LES tests with acute nicotine are presented in Figures 1-6.

Overall Mean LES. Overall session mean difference score data are presented in Figure 1. As suggested by the difference score data from Figure 1, nicotine suppressed LES in males but not females. A Drug X Sex ANOVA revealed a main effect of Drug, $F(2, 140) = 4.48$, $p < .01$, and a significant Drug X Sex interaction, $F(2, 140) = 6.49$, $p < .01$. Separate one-way ANOVAs conducted on data from males and females separately revealed a main effect of Drug for males $F(2, 70) = 7.62$, $p \leq .001$,but not for females $F(2, 140) < 1$. In males, nicotine significantly suppressed LES at both 0.15 mg/kg and 0.40 mg/kg doses, $Fs(1, 70) \geq 8.83$, $ps < .01$.

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Insert Figure 1 about here

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Within-Session Changes. In order to explore temporal factors in nicotine's impact on LES, data from test session were blocked into the first half of the session (trial block 1-15) and the second half of the session (trial block 16-30). For reference, the midpoint of trial 1-15 is approximately 14 min post-nicotine injection, and the midpoint of trial 16-30 is approximately 20 min post-nicotine injection. A Drug X Block X Sex ANOVA conducted on difference score data from the test revealed a significant main effect of Drug $F(2, 140) = 4.48$, $p \leq .01$.as well significant Drug X Block, $F(2, 140) = 5.83$, $p \leq .01$, Drug X Sex, $F(2, 140) = 6.49$, $p \leq .01$, and Block X Sex, $F(1, 140) = 3.98$, $p \leq .05$, interactions. As can be seen in Figure 2 for males, nicotine at both doses had a generally suppressive effect on LES. A Drug X Block ANOVA conducted on difference score data for males revealed only a main effect of Drug, $F(2, 70) = 7.63$, $p \leq .001$. Difference scores in 0.15 mg/kg and 0.40 mg/kg nicotine conditions were significantly lower than in the Sal condition at both trial 1-15, $Fs(1, 70) \geq 23.96$. $ps < .001$, and trial 16-30, $Fs(1, 70) \geq 5.19$. $ps < .05$, periods of the session. Figure 3 displays LES data from females. As suggested by Figure 3, females were more resistant to the suppressive effect of nicotine on LES than males. A Drug X Block ANOVA conducted on test data from females revealed a significant effect of Block $F(1, 70) = 9.92$, $p < .01$and a significant Drug X Block interaction $F(2, 70) = 5.65$, $p < .01$. The effect of Block appears to be in the tendency for LES to increase across blocks in the 0.40 mg/kg nicotine condition. Difference scores at the trial 16-30 block were significantly higher in the 0.40 mg/kg nicotine condition than in the sal condition, $F(1, 70) = 5.40$, $p \leq .05$, indicating that 0.40 mg/kg nicotine was anxiogenic approximately 20 min following administration but not anxiogenic during earlier parts of the session.

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Insert Figures 2 and 3 about here

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Overall Mean LES: First Drug Test. Order of drug testing was carefully counterbalanced and 48 hours intervened between test sessions. Nonetheless we sought to explore whether patterns of findings observed above would generally be maintained upon examination of data from only the first drug test (Drug Test 1) which is uncontaminated from any prior drug exposure. Overall session mean difference score data from Drug Test 1 of Experiment 1 are presented in Figure 4. A Drug X Sex ANOVA conducted on the difference scores from Test 2 revealed a significant Drug X Sex interaction, $F(2, 66) = 3.05$, $p \leq .05$. One-way ANOVAs conducted on difference score data separately for males and females revealed a significant main effect of Drug for males, $F(2, 33) = 3.36$, $p \leq .05$, but not for females, $F(2, 33) < 1$. As suggested

in Figure 4 for males nicotine at both doses impaired LES although this difference was significant only at the 0.15 mg/kg nicotine dose, $F(1, 33) = 6.71$, $p \leq .05$.

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Insert Figure 4 about here
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Within-Session Changes: First Drug Test. Temporal factors in nicotine's impact on LES was assessed by blocking data from the post-nicotine test session into the first half of the session (trials 1-15) and the second half of the session (trials 16-30). Again for reference the midpoint of trial 1-15 is approximately 14 min post-nicotine injection, and the midpoint of trial 16-30 is approximately 20 min post-nicotine injection. A Drug X Block ANOVA conducted on data from males revealed a significant main effect of Drug $F(2, 33) = 3.36$, $p \leq .05$.as well as a significant Drug X Block interaction, $F(2, 33) = 6.64$, $p \leq .01$. As can be seen in Figure 5 for males, nicotine at both doses impaired LES early in the test session (trial 1-15) but later in the test session (trial 16-30) nicotine's suppressive effect on LES was maintained at the 0.15 mg/kg dose but not at the 0.40 mg/kg dose. Comparison of LES across early and late potions of the session thus reveals LES was released from nicotine's suppressive effect at the 0.40 mg/kg dose. In the first half of the session (trial 1-15) LES in nicotine animals was significantly lower than saline at both 0.15 mg/kg and 0.40 mg/kg doses, $Fs(1, 33) > 14.28$, $ps < .01$. During the second half of the session (trial 16-30) LES was significantly lower compared to saline at the 0.15 mg/kg dose, $F(1, 33) = 8.61$, $p < .01$, but not at the 0.40 mg/kg dose, $F(1, 33) < 1$. Finally, LES was significantly higher in the second half of the session compared to the first half of the session in the 0.40 mg/kg group, $F(1, 33) = 14.95$, $p < .01$. No main effect or interaction as significant in females, $Fs < 1$, although there was a small tendency for nicotine at the higher dose to suppress LES in the early part of the session.

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Insert Figures 5 and 6 about here
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Experiment 2

The main finding from Experiment 1 was that at a short 5 min nicotine-to-test interval nicotine was generally anxiolytic in males at both .15 mg/kg and .40 mg/kg doses. Interestingly in females, within-session variation of startle revealed nicotine was anxiogenic at the high .40 mg/kg dose, but only during the second half of the test session (Figure 3). This latter finding is noteworthy because it suggests a time-dependent progression toward anxiogenic profile as time since nicotine administration increases. Data from males on the first drug test only are further consistent with this tendency. As illustrated in Figure 5, .40 mg/kg nicotine was anxiolytic during the first half of the test session, an effect which was progressively lost by the second half of the test session. It can be noted that the midpoint of the overall LES test session is approximately 15 min following nicotine administration. Collectively, these findings reveal a pattern toward a progressing anxiogenic profile of 0.40 nicotine in LES at longer post-nicotine intervals, especially in the time frame of greater than 15 min post nicotine injection. The goal of Experiment 2 was to explore the effect of nicotine on anxiety at longer post-nicotine intervals by further exploring time-dependent effects of nicotine on LES. Because Experiment 1 revealed a progressing anxiogenic trend only at .40 mg/kg dose, only this dose was used in Experiment 2.

The half-life of nicotine has been reported to be 51.4 minutes (Jung et al., 2001). With this pharmacodynamic of nicotine in mind, Experiment 2 explored the impact of nicotine on LES at two longer nicotine-to-test intervals than in Experiment 1: 15 min and 35 min. These two intervals were selected because within the actual LES test session, after animals are loaded in chambers, there is a 5-minute no-stimulus acclimation period before startle eliciting noise stimuli are initiated and the total duration of actual startle probe testing is 15 min once initiated. Therefore, a group with a 15 minute nicotine-to-test interval (followed by the 5 min session acclimation period) would have their startle reactivity measured 20-35 minutes following nicotine administration. Similarly, a group with a 35 minute nicotine-to-test interval (followed by the 5 min session acclimation period) would have their startle reactivity measured 40-55 minutes following nicotine administration. In this way, Experiment 2 permits systematic assessment nicotine's impact on anxiety at different time periods within nicotine's pharmacological half-life.

In Experiment 2, saline or .40 mg/kg (i.p) nicotine was administered to male and female rats either 15 min prior to LES testing or 35 min prior to LES testing. The interest of this part of the experiment was in whether nicotine would be anxiogenic at longer nicotine-to-test intervals than the interval used in Experiment 1. It should be noted that any findings from Experiment 2 must be viewed as preliminary for two reasons. First, data presented as Experiment 2 reflect an incomplete and only partially counterbalanced replication. Counterbalancing of sex, order of drug test and other factors were conditioned on a full balanced replication which did not occur owing to William & Mary campus restructuring associated COVID-19 in Spring 2020. Second, additional follow up studies planned were cancelled and not conducted. Nevertheless, limited analyses and examination of trends in Experiment 2 are supportive of findings from Experiment 1 and grounds for further study.

Method

Subjects

Thirty-six Sprague-Dawley rats (18 male and 18 female) approximately 90 days old were used. Animals were maintained as in Experiment 1.

Apparatus

The apparatus was as described in Experiment 1.

Procedure

Nicotine. Nicotine bitartrate dihydrate salt was used to create nicotine injection solutions as described in Experiment 1 except that only the 0.40 mg/kg nicotine dose was used in Experiment 2.

LES Test Procedure. The LES test procedure was identical to that of Experiment 1 with exceptions noted below.

*Baseline LES and Nicotine Challenge LES Test Procedure*s. Baseline LES scores were recorded during a preliminary drug-free LES test exactly as in Experiment 1. Rats were assigned to two different (between-subjects) drug groups (saline or 0.40 mg/kg nicotine) counterbalanced as closely as possible according to baseline LES, sex, and weight. Nicotine challenge in LES testing occurred as in Experiment 1 except that in Experiment 2 each animal received two as opposed to three nicotine challenge tests counterbalanced as closely as possible. In one of the two nicotine LES test conditions, nicotine (or saline) was administered at the beginning of a 15 minute wait interval that separated Phase 1 (dark) and Phase 2 (light). In the other nicotine LES test condition, nicotine (or saline) was administered at the beginning of the 35 minute wait interval that separated Phase 1 (dark) and Phase 2 (light).

Results and Discussion

Overall Mean LES. Overall session mean difference scores are presented in Figure 7. As can be seen in Figure 7, 0.40 mg/kg nicotine did not generally impact LES in either males or females. A Delay X Drug X Sex ANOVA conducted on overall means of difference score data failed to reveal any significant effect or interaction, all Fs $\leq$ 1.41.

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Insert Figure 7 about here

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Within-Session Changes. For symmetry of analysis as in Experiment 1, data from Experiment 2 were examined in order to explore within-session changes in LES by blocking data into the first half of the session (trials 1-15) and the second half of the session (trials 16-30). A Delay X Drug X Block X Sex ANOVA revealed only a significant Block X Sex interaction, $F(1, 32) = 15.83$ $p < .001$. As suggested by Figures 8 and 9 there was a tendency for difference scores to decrease across the session for males but increase across the session for females.

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Insert Figures 8 and 9 about here

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Overall Mean LES: First Drug Test: For further symmetry of analysis with Experiment 1, data from the first drug test (uncontaminated by effects of prior drug exposure) were subjected to a Delay X Drug X Sex ANOVA. A Delay X Drug interaction approached statistical significance ($p = .067$). Remaining results are presented in graphical form only without analysis due to insufficient sample size. As can be seen in Figure 10, there was a consistent tendency toward an anxiogenic effect of nicotine was seen in both males and females in the 15 min delay condition but not in the 35 min delay condition. Within-session changes for the first drug test only were not assessed.

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Insert Figures 10, 11, and 12 about here

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Overall Mean LES: Comparison Across Experiments. Comparison of data from Figure 7 (all tests included) and Figure 10 (first drug test only) suggests a stronger overall anxiogenic effect of nicotine on the first drug test (Figure 10) compared to the aggregate of all tests together (Figure 7). A similar trend can be seen from Experiment 1 by comparison of males' difference score data in Figure 1 (all tests included) to those of Figure 4 (first drug test only, at least in some nicotine conditions). Given this pattern, an overall summary of trends across experiments is provided next that includes the overall session mean from the first drug test only. Because only saline and 0.40 mg/kg groups were run in both experiments, these groups will be illustrated. These data are presented in Figures 11 and 12 for comparison. A consistent time-dependent pattern emerges at the two shorter post-drug delay intervals. In males, administering nicotine 5 min prior to behavioral testing impaired LES but at a longer 15 min post-nicotine interval nicotine administration enhanced LES (Figure 11). Interestingly, females (Figure 12) were more resistant to nicotine-impairing effects on LES at the 5 min wait interval but consistent and

similar to males nicotine enhanced LES at the 15 min wait interval. An exploratory Delay X Drug X Sex analysis including the shorter two interval conditions (5min, 15min) revealed a significant Delay X Drug interaction, $F(1, 56) = 4.51$, $p = .038$. At the longest post-nicotine interval (35 min) in both males and females nicotine did not enhance LES.

General Discussion

These experiments sought to examine the relationship between nicotine dose and anxiety measured in the startle paradigm of LES while further attempting to extrapolate a relationship between delay of nicotine administration and anxiety behavior. Importantly, the effects of nicotine in the LES model of anxiety has not been well explored to date. Experiment 1 found that at a short 5 min drug-to-test interval nicotine suppressed LES (an anxiolytic profile) in males. Males at both nicotine doses tested (.15 mg/kg and .40 mg/kg) displayed lower LES compared to saline controls. This reveals an important point of contrast to anxiety measured in the social interaction test by Irvine et al. (1999) who reported nicotine was anxiogenic in male rats with a short 5 min nicotine-to-test interval using .1 mg/kg nicotine. Interestingly, a similar anxiogenic profile of nicotine at the same short 5 min nicotine-to-test interval was reported by Biala & Budzynska (2005) in the elevated plus maze, who also used .1 mg/kg nicotine. This contrast of other research demonstrating that relatively immediate nicotine increases anxiety also persists at higher nicotine doses. File et al. (2002) reported that .45 mg/kg nicotine was anxiogenic in the social interaction test, again, with a 5 min drug-to-test interval. Therefore, differences in nicotine dose between Irvine et al. (1999) and the present research may not explain the differences in outcome.

One alternative theory to the apparent anxiolytic effect of nicotine seen in Experiment 1 is that nicotine suppressed motor activity during the startle test and did not suppress anxiety. Without further experimental assessment, this theory remains possible. There is evidence, however, to suggest that the motor suppressive effects of nicotine cannot adequately explain reduced LES in nicotine groups of Experiment 1. In the social interaction test, File et al. (1998) reported that .1 mg/kg and .5 mg/kg nicotine doses that were capable of modifying anxiety (prosocial) behavior did not affect motor activity; motor suppressive effects due to nicotine were seen only at the highest dose of 1.0 mg/kg. Irvine et al. (2001) reported that nicotine delivered directly and bilaterally to the dorsal hippocampus at doses sufficient to affect anxiety behavior in the social interaction test had no effect on motor activity. Additionally, File et al. (2002) argued that moderate .45 mg/kg doses of nicotine similar to that of the high-dose condition used in the present experiments do not affect motor activity. Bryson et al., (1981) reported in rats tested for general activity (wheel running) shortly after administration of .2 mg/kg and .4 mg/kg nicotine, doses similar to those of Experiment 1, that nicotine produced motor enhancing and not motor suppressive effects. Meliska, Fitzpatrick, & Rosine (1974), however, reported that .1 mg/kg and .4 mg/kg nicotine can have motor suppressive effects in rats that dissipate after approximately 20 min. Even here, however, reported motor suppressive effects of nicotine at these doses were not uniform and were not present in all groups, especially those with modest pre-experiment baseline motor activity levels. With nicotine delivered at longer delays, Khalki et al. (2014) reported that .5 mg/kg nicotine administered 35 min before testing enhanced (not suppressed) locomotor activity in the open field.

Experiment 1 further permitted sex comparisons. In Experiment 1, an anxiolytic profile for nicotine was suggestive for males at both nicotine doses tested but was not anxiolytic in females at either dose. In fact, within-session variation in LES revealed the higher .40 mg/kg nicotine dose for females was anxiogenic compared to saline controls, at least in latter parts of

the session (Figure 3). Sex-dependent effects were seen in Experiment 1 both when all nicotine tests were considered together (Figures 1-3) and when only Test 1 (the first drug test) was considered (Figures 4-6). These data indicate that male and female rats are affected by nicotine in different ways.

The idea that male and female rats process nicotine differently has been suggested by animal studies which have implicated that nicotine's anxiogenic or anxiolytic effect can be dependent on gender (see Morissette et al., 2007 for a broad review). For example, Donny et al. (2000) showed that in a self-administration paradigm female rats had higher motivation to work for nicotine than male rats suggesting male and female rats are not only affected by nicotine differently but may metabolize nicotine at different rates affecting nicotine reinforcement. Interestingly, this has been corroborated on a molecular level where it was found that male rats have a metabolic rate of nicotine at a 3 to 5 fold higher rate when compared to females (Craig et al., 2014). Differences in nicotine metabolism between males and females do not, however, provide a likely account of sex differences in LES seen in these experiments. Slow nicotine metabolism in females would anticipate stronger psychoactive properties (nicotine remains active at fuller 'concentrations') and therefore females might show a *stronger* anxiety modulating effect of nicotine compared to males, whether anxiolytic or anxiogenic. Yet in Experiment 1, nicotine's effect on anxiety was generally stronger and more consistent for males compared to females. Slower nicotine metabolism in females would further anticipate *longer lasting* psychoactive properties of nicotine and therefore less within-session variation of LES anxiety (nicotine's active state remains more stable and durable over time). Following this same logic, males would be expected to show a comparatively stronger within-session variation in LES anxiety (greater presumed difference in nicotine availability in early compared to late parts of the session). In Experiment 1 within-session variation in LES anxiety was revealed both for females (Figure 3) and for males (Figure 5) but with no systematic sex effect. Sex differences in nicotine metabolism are not likely the cause of observed differences in how nicotine differently affected LES in males and females in Experiment 1.

The exact mechanism through which nicotine may produce different effects in males and females is not understood (Cross, Linker, & Leslie, 2017). However, one possibility is that nicotine could have differing effects in the BLA in male and female rats. Gonadal hormones such as estrogen and progesterone may play important roles because they act as allosteric modulators of GABA, glutamate and nAChRs (Cross, Linker, & Leslie, 2017). The influence of these hormones on GABA is of interest because GABA has been implicated in modulating anxiety. For example, infusions of GABA agonists into the amygdala in mice has shown decreased anxiety in the elevated plus maze, while infusing a GABA antagonist had an anxiogenic effect (Nuss, 2015). Varani and Blaerio (2012) further demonstrated $GABA_B$ receptor involvement in nicotine mediated anxiogenic or anxiolytic behavior. Importantly, how GABA is transmitted and regulated can be different between sexes due to gonadal hormones. Estradiol, for example, has been found to reduce GABA neurotransmission and increase dendritic spine density producing higher likelihood of excitatory post-synaptic potentials in the rat hippocampus (Murphy et al., 1998). If estrogens acted similarly to reduce activation of GABAergic pathways in the BLA this could produce a less anxiolytic and more anxiogenic profile of nicotine in females compared to males, exactly the pattern of outcomes observed in Experiment 1.

In both sexes from Experiment 1, which used a short 5 min nicotine-to-test interval, there were within-session changes in LES anxiety with later parts of the session expressing a more strongly anxiogenic tone (or less an anxiolytic tone). Additionally, Irvine et al. (1999) reported different effects of nicotine on anxiety at longer compared to shorter nicotine-to-test intervals in the social interaction test. Therefore Experiment 2 examined the effect of longer 15 min and 35

min nicotine-to-test intervals on LES anxiety. Some suggestive trends emerged. Among male and female rats there was a common pattern observed which showed that nicotine 15 minutes post injection had an anxiogenic-like profile but at 35 minutes post nicotine had an anxiolytic-like profile (Figure 10). This pattern of nicotine increasing LES at a 15 minute delay and decreasing LES at a 35 minute delay that was found in both male and females is suggestive that nicotine's effect on anxiety was time dependent. This claim (like others for Experiment 2) cannot be made with certainty due to lack of adequate statistical analysis.

Comparisons across Experiments 1 and 2 in the .4 mg/kg nicotine condition similarly reveal consistent patterns (Figures 11 and 12). At the 5 minute delay, LES in nicotine treated conditions was lower when compared to saline controls (an effect stronger in males), but at the 15 min delay LES was higher (in both males and females) and at the longer 35 min delay LES was generally lower in nicotine treated animals. This suggests nicotine is anxiolytic at shorter drug-to-test intervals (5 min), anxiogenic at moderate drug-to-test delays (15 min) and (possibly) anxiolytic at longer delays (35 min). These results described for .4 mg/kg nicotine, specifically at short 5 min drug-to-test delay, are in direct opposition to those reported by Irvine et al. (1999) in the social interaction test. Irvine et al. (1999) found that .1 mg/kg nicotine produced anxiogenic effects 5 min post nicotine but produced anxiolytic effects 30 min post nicotine. The difference between anxiogenic (social interaction test) and anxiolytic (LES) outcomes at a short interval cannot be explained by differences in drug dose because Experiment 1 similarly found an anxiolytic effect of nicotine at the short 5 min delay using .15 mg/kg nicotine, similar to the nicotine dose employed by Irvine et al. (1999). The basis for this difference is not clear but the reflexive (LES) and non-reflexive (social interaction test) requirements of the tasks suggest the engagement of different brain systems which may be differentially responsive to nicotine.

The relatively immediate anxiolytic effect of nicotine observed in 'reflexive' LES anxiety is important in part because it differs from the relatively immediate nicotine-induced anxiogenic effect in the 'non-reflexive' social interaction test and elevated plus maze. This implies a fundamentally different time course of nicotine's anxiolytic and anxiogenic profile in reflexive and non-reflexive measures of fear. The immediate anxiolytic effects of nicotine observed in Experiment 1, which utilized LES, are further important for other reasons. Smokers often report they smoke because smoking *reduces* their anxiety level (Parrott, 1999) and thus understanding immediate anxiolytic effects of nicotine is crucial to understanding factors that promote maintenance and relapse of nicotine addiction. The finding that nicotine produced immediate anxiolytic effects in LES may suggest LES as a powerful and possibly more valid animal model for examining mechanisms through which nicotine reinforces addictive behavior. Collectively, the present experiments establish and demonstrate that the LES paradigm is sensitive to nicotine-induced alterations in anxiety and is at least suggestive that this paradigm is further sensitive to temporally dynamic effects of nicotine on behavior.

Future Directions and Follow-up Experiments that were cancelled owing to COVID-19. Eliminating the motor suppression hypothesis for nicotine-induced suppression of LES seen in Experiment 1 could be directly addressed by replicating the procedure of Experiment 1 under conditions in which both phases of the LES test session are conducted in the dark (as opposed to the first session in dark and the second session in light, as occurred in Experiment 1 and 2). If nicotine at the doses tested suppresses startle (motor) reactivity, significantly lower startle means should occur in the second (nicotine exposed) dark session compared to the first (nicotine-free) dark session. If nicotine affects anxiety and not startle (motor) reactivity per se, then startle reactivity should be the same in the first (drug free) dark session and second (nicotine present) dark session both of which do not occur with the anxiety-inducing light. If nicotine does not

affect motor function, nicotine would not be expected to affect startle per se, but only affect startle in the presence of an environmental stressor (light).

Other follow up experiments include adding a .15 mg/kg nicotine group to Experiment 2 varying drug-to-test delay that would have allowed stronger comparison to available related research using the social anxiety test and elevated plus maze anxiety paradigms.

In general, experiments that have explored an aspect of time's effects on nicotine suggest that drug-to-test intervals play a role in the modulatory effect of nicotine on anxiety (Irvine et al., 1999; Biala & Budzynska, 2005). Findings from the present research and that of others are important to the field as a whole. Data from LES and other paradigms implicates that drug-to-test delays can have profound effects on anxiety state, because the same dose can have different effects at different times (Irvine et al., 1999; Experiments 1 and 2 presented here). As such, future research into nicotine's anxiogenic (or anxiolytic) profile should take into account the temporal dynamics of nicotine's impact when selecting experimental parameters.

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Figure Captions

Figure 1. Overall session mean difference scores in males and females from Experiment 1. Brackets represent standard errors.
Figure 2. Mean difference scores in males during the first and second half of the test session from Experiment 1. Brackets represent standard errors.
Figure 3. Mean difference scores in females during the first and second half of the test session from Experiment 1. Brackets represent standard errors.
Figure 4. Overall session mean difference scores in males and females from Test 1 of Experiment 1. Brackets represent standard errors.
Figure 5. Mean difference scores in males during the first and second half of the test session from Test 1 of Experiment 1. Brackets represent standard errors.
Figure 6. Mean difference scores in females during the first and second half of the test session from Test 1 of Experiment 1. Brackets represent standard errors.
Figure 7. Overall session mean difference scores in males and females from Experiment 2. Animals received either a 15-min or a 35-min drug-to-test interval. Standard error bars are omitted due to small sample size.
Figure 8. Mean difference scores in males during the first and second half of the test session from Experiment 2. Animals received either a 15-min or a 35-min drug-to-test interval. Standard error bars are omitted due to small sample size.
Figure 9. Mean difference scores in females during the first and second half of the test session from Experiment 2. Animals received either a 15-min or a 35-min drug-to-test interval. Standard error bars are omitted due to small sample size.
Figure 10. Overall session mean difference scores in males and females from Test 1 of Experiment 2. Animals received either a 15-min or a 35-min drug-to-test interval. Standard error bars are omitted due to small sample size.
Figure 11. Overall session mean difference scores in males from Test 2 of Experiment 1 and Experiment 2. Across experiments, animals received a 5-min, 15-min, or a 35-min drug-to-test interval. Standard error bars are omitted due to small sample size.
Figure 12. Overall session mean difference scores in females from Test 2 of Experiment 1 and Experiment 2. Across experiments, animals received a 5-min, 15-min, or a 35-min drug-to-test interval. Standard error bars are omitted due to small sample size.

APPENDIX A: Literature Review

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

Literature Review

Topic: Light Enhanced Startle
Summary: Light Enhanced startle is associated with anxiety

Walker, D. L., & Davis, M. (1997). Anxiogenic effects of high illumination levels assessed with the acoustic startle response in rats. *Biological Psychiatry*, *42*(6), 461–471. doi: 10.1016/s0006-3223(96)00441-6

The study at hand aimed to quantify the effects of high illumination on rats through the acoustic startle response. Previous studies showed that when rats were in environments of high illuminance, they released more corticosterone. An important point that was brought up, was that previous anxiety models (FPS) rely heavily on conditioned responses which makes testing anxiolytic drugs difficult from this model because the model itself relies on learning. For the first experiment the researchers examined the startle amplitude of rats in an illuminated test chamber when compared to the startle amplitude of rats in a darkened test chamber. Here researchers measured the startle of rats in groups that had either an 8-, 70- or 700- footlambert light. Each group of rats were tested once on two different days. On the first day, rats startle was measured in the dark during phase I and in the light during phase II. A day later the same rats were placed in a dark to dark session and the amplitudes of their startle responses were measured. Here the researchers found that startle was greater in the presence of light with the 70- and 700-footlambert light but not the 8- footlambert light. The researchers thought that the change of illumination levels from dark to light might have been responsible for increases of startle, so they performed the same experiment except on day 2, they made the phase I of LES light and the second phase of LES dark. Here the researchers found that whenever illumination was lowered, startle decreased.

The second experiment examined how acoustic startle correlated with anxiety. Here the researchers had four groups of rats: dark phase I to light Phase II with saline, dark phase I to light phase II with buspirone, dark phase I to dark phase II with saline, and dark phase I to dark phase II given buspirone. Here the light phases had a 700-footlambertlight. Buspirone was given because it is an anxiolytic drug that was shown to block anxiety in rats in other paradigms. The results showed that the LES was blocked by buspirone, indicating a connection between anxiety and LES.

Experiment 3 examined that habituation of LES by testing rats 32 times over 2 weeks. For the first 16 sessions rat's LES was tested using a dark phase I to dark phase II LES. For the next 16 sessions rats were tested using the dark phase I to light phase II LES. The light used here was the 700-footlambert light. The results of this indicated that LES had little habituation. In general, the researchers found that LES was enhanced in the presence of light and suggested that LES was more linked to measuring anxiety than FPS because LES relies on an unconditioned stimulus and allows for easy interparticipant examination.

Topic: Light Enhanced Startle
Summary: LES is correlated with anxiety which is different than Fear Potentiated Startle

Jongh, R. D., Groenink, L., Gugten, J. V. D., & Olivier, B. (2003). Light-enhanced and fear-potentiated startle: temporal characteristics and effects of α-helical corticotropin-releasing hormone. *Biological Psychiatry*, *54*(10), 1041–1048. doi: 10.1016/s0006-3223(03)00468-2

The study at hand examined the time course of LES and FPS and investigated whether corticotropin-releasing hormone (CRH) is differentially involved in these two models. The first part of the experiment used LES and FPS on rats to determine anxiety state. For LES researchers used LES with three phases. In the beginning of phase I and phase II there was a 5 minute acclimation period but phase III did not have this. Here the researchers tested male rats with 3 times, under 3 different conditions with each test day being separated by 72 hours. In condition 1 the light remained off in all three sessions, in condition 2 phase I was dark but phase II & III had a 900 lux bulb on, and condition 3 only phase II was illuminated. Also, a separate group of male and female rats were tested in a fear potentiated startle paradigm twice. Here the researchers found that when the light was turned on, LES was potentiated. FPS revealed there to be no differences between male and female rats.

In the second experiment male rats were tested in LES twice a week for 4 weeks. On one of these test days, the light was off in both phases but on the other day a 900 lux bulb was on during phase 2. Here rats were given either 0, 1,5, or 25 ug of alpha-helical CRH antagonist. A separate group of male rats was trained to perform FPS just like the first experiment but one day after the last training the animals received one of the previous doses listed. Here the researchers found that α -helical CRH reduced the potentiation in LES, but not in FPS. The researchers suggested that this was difference of effect on two different paradigms of anxiety was because each paradigm measures different emotions. The researchers then pointed out that LES was more correlated to anxiety due to the time course of the paradigm and that FPS was more correlated due to fear and this was corroborated by previous research about the molecular involvement of CRH in different emotions.

Topic: Nicotine Induced Anxiety
Summary: Chronic nicotine exposure can lead to increased levels of anxiety

Slawecki, C., Gilder, A., Roth, J., & Ehlers, C. (2003). Increased anxiety-like behavior in adult rats exposed to nicotine as adolescents. *Pharmacology Biochemistry and Behavior*, *75*(2), 355–361. doi: 10.1016/s0091-3057(03)00093-5

This study aimed to examine the relationship between anxiety following adolescent behavior. For this test rats at 28 days were exposed to nicotine for 5 days (5.0 mg/kg/day nicotine patches) while others were shaved and given a band aid in order to replicate the feeling of a transdermal patch on control rats. During this time of nicotine exposure motor activity of the rats was monitored for the first 4 days and also 10 days after their last exposure to nicotine. After 5 days, the nicotine was removed from the nicotine rats, and the band aids were removed from the control rats. Two weeks after the rats last exposure to nicotine, the control and nicotine rats were tested. For the testing, the experimenters placed rats in a standard open field test and a modified open field test which had food in the center.

The results showed that rats exposed to nicotine had lower motor function, fewer perimeter square entries and total square entries (standard open maze) and less interactions with the food in the center of the modified open maze test. The researchers also noted that the rats exposed to nicotine had less exploratory behavior. The researchers noted that lack of interactions and entries indicated states of higher anxiety, but they could not confidently trust this because they speculated that this lack of interaction may have been due to the withdrawal of nicotine.

Topic: Nicotine Induced Molecular Changes
Summary: Chronic nicotine exposure can lead to a changed neurophysical response to fear

Slawecki, C. J., & Ehlers, C. L. (2003). The effects of corticotropin-releasing factor on the cortical EEG are reduced following adolescent nicotine exposure. *Neuropeptides*, *37*(1), 66–73. doi: 10.1016/s0143-4179(03)00006-4

This study was done to assess the effects of adolescent nicotine on the cortical and hippocampal EEG responses to ICV corticotropin-releasing factor (CRF) in order to determine if adolescent nicotine exposure impacts adult CRF systems. In the study, nicotine rats were given 5 mg/kg/day of nicotine via a transdermal nicotine patch for 5 days in adolescents. Control rats were given band aids to replicate the feel of the patch for 5 days. After 5 days nicotine administration was stopped. It should be noted here, that all rats in the experiment were male. Brains were then taken to perform EEG tests 4 to 5 weeks after the nicotine exposure ended. Prior to EEGs being performed rats were injected with .10ug, .50ug, and 1.0ug CRF. CRF was administered because previous research showed that infusion of CRF induces behavioral signs of anxiety. When performing the EEG recordings were taken 10 minutes after the CRF was administered and then recordings took place over 20 minutes. EEG's were recorded in the frontal cortex, parietal cortex, and hippocampus. The results of this experiment indicated that the effects of CRF on the cortical EEG were diminished in rats exposed to nicotine when compared to control rats. This data indicates that chronic nicotine exposure decreases the neurophysical responds to exogenously administered nicotine. The researchers mentioned that this may have occurred because chronic nicotine exposure diminishes neuro physical responses to exogenously CRF. This is important because previous research has suggested that chronic nicotine exposure can be characterized by a decreased neurophysical response to stress. The researchers then hypothesized that these results may have happened because prolonged stimulation of the CRF systems during nicotine exposure induces a relatively long-lasting decreases in sensitivity of cortical systems to CRF.

Topic: Chronic Nicotine Exposure
Summary: Chronic nicotine exposure can lead to increased spatial memory

Levin, E. D., Lee, C., Rose, J. E., Reyes, A., Ellison, G., Jarvik, M., & Gritz, E. (1990). Chronic nicotine and withdrawal effects on radial-arm maze performance in rats. *Behavioral and Neural Biology*, *53*(2), 269–276. doi: 10.1016/0163-1047(90)90509-5

This study aimed to see whether chronic nicotine administration would improve choice accuracy in a non-stressful cognitive task. More specifically, the researchers examine how chronic nicotine administration would affect rat's choice accuracy and locomotor speed in the radial arm maze test. In order to ensure the accuracy of the experiment, rats were ran twice on the radial arm test. It should also be noted that the radial arm test had food on each wing and that the rats were kept on a restricted food diet. Rats also underwent at least 20 training sessions before the experiment began, during the experiment rats were tested on the maze 2 times per week. Nicotine in this study was administered via a subcutaneously implanted glass and silastic pellet, which gave nicotine blood levels of 400-500 ng/ml. Rats that were not administered with nicotine underwent the same surgery of the silicon beads that nicotine rats underwent and had the silica beads removed at the same as the nicotine rats (3 weeks). The difference between the control rats and the nicotine rats was that the control group had silica pellets that did not contain nicotine. Interestingly, this experiment only tested female rats.

Testing went on for 5 weeks, the first three weeks, nicotine rats received nicotine and at week 4 nicotine administration stopped; their results were then compared to the results of the control rats. The results showed that chronic nicotine administration improved the spatial memory test, and that this improvement was also delayed until the second week of receiving nicotine. Additionally, the effects of nicotine were still present when nicotine was withdrawn from the rats. The researchers hypothesized that these effects occurred were due to the fact that chronic nicotine exposure leads to an additional period of learning.

Topic: Withdrawal from Chronic Nicotine
Summary: Withdrawal from nicotine can cause decreased fear conditioning

Davis, J. A., James, J., Siegel, S., & Gould, T. (2005). Withdrawal from Chronic Nicotine Administration Impairs Contextual Fear Conditioning in C57BL/6 Mice. *Journal of Neuroscience*, *25*(38), 8708–8713. doi: 10.1523/jneurosci.2853-05.2005

Nicotine withdrawal has been shown to be associated with cognitive defects. It was found that initially nicotine has an enhancing effect on learning and memory which is reinforced by repeated use, and chronic use is done to avoid nicotine withdrawal because nicotine withdrawal induced cognitive deficits. Here, the researchers wanted to examine how nicotine withdrawal could influence fear conditioning. In this experiment, the researchers administered nicotine to rats via micro- osmotic pumps that would give 6.3 mg/kg/d of nicotine or saline. Here rats received treatment for 14 days, while being trained in the fear conditioning model at day 13 and being tested in the model on day 14. Mice in the withdrawal group, were given doses of saline or nicotine for 12 days and then stopped receiving doses. Next the withdrawal mice were then trained in the fear conditioning model on day 13 with testing on day 14. Additionally, the researchers performed a follow up study where they saw if acute nicotine administered during fear conditioning in the withdrawal group could reverse any effects. Using a typical fear conditioning model, the researchers found that acute nicotine administration to the withdrawal group significantly enhanced contextual fear conditioning when compared to all other treatment groups. Additionally, the researchers noted that there was no effect of chronic nicotine administration on contextual fear conditioning. Also, mice that were chronically given saline and not acute nicotine before training demonstrated higher levels of contextual fear than saline mice. The researchers found that the mice withdrawn from nicotine had the worst fear conditioning, and that an administration of an acute dose of nicotine reversed these effects. The results lead to the researchers discussing that the percentage of contextual freezing in mice withdrawn from chronic nicotine treatment that had received acute nicotine was similar to the percentage of contextual freezing in mice withdrawn from chronic saline treatment that also received acute nicotine. The results however were unclear and made the researchers question whether the percentage increase in contextual conditioning in mice withdrawn from nicotine reflected sensitization to the effects of nicotine or a reversal of the withdrawal associated deficit. Additionally, the researchers found that acute and chronic nicotine produce the same plasma nicotine levels but not same effect: acute nicotine enhanced fear conditioning while chronic nicotine exposure did not.

Topic: Molecular basis of nicotine
Summary: Nicotine has a molecular basis to improve memory via LTP
Note: this is a review that focuses on nicotine, and drugs that act on nAChR receptors, my interest in this annotated bibliography will be on nicotine and general nAChR processes

Buccafusco, J., Letchworth, S., Bencherif, M., & Lippiello, P. (2005). Long-lasting cognitive improvement with nicotinic receptor agonists: mechanisms of pharmacokinetic–pharmacodynamic discordance. *Trends in Pharmacological Sciences*, *26*(7), 352–360. doi: 10.1016/j.tips.2005.05.007

It was first noted by studies in the 1990s that nicotine, an nAChR agonist, improved cognition for prolonged periods of time in non-human primates. In fact, it was found that nicotine was able to enhance cognition after discontinued use in rats. This phenomenon was first thought to occur due to a metabolite of nicotine called cotinine. This, however, was shown not to be upheld because one study showed that rats' performance in the radial arm maze increased long after nicotine and cotinine had been in the body. This same effect was also found to not be unique to nicotine. Instead this effect was also found in other nACHR agonists. This has thus led to the author's proposed mechanism of how nicotine can increase cognitive performance via long term potentiation (LTP).

LTP correlates with memory, and as this process occurs, memory consolidation follows suit. When LTP first occurs in the early stages, Ca ion dependent systems alter protein composition in order to increase neurotransmitter release from presynaptic neurons and support short-term memory. Later phases of LTP require prolonged elevation of intraneuronal levels of Ca and protein synthesis

> *"The latter activates a cascade that involves adenylyl cyclase, cAMP, and secondary messengers such as protein kinase A and MAPK. In turn these enhance the activity of the cAMP response element-binding protein 1 (CREB-1) transcription factor, which increases the expression of immediate early genes and stimulates the synthesis of growth factors and other proteins that maintain cell excitability and support the formation of new synaptic connections".*

Topic: Pharmacokinetics of Nicotine
Summary: Nicotine base and nicotine salt have different half-lifes

Jung, B. H., Chung, B. C., Chung, S.-J., & Shim, C.-K. (2001). Different pharmacokinetics of nicotine following intravenous administration of nicotine base and nicotine hydrogen tartarate in rats. *Journal of Controlled Release*, *77*(3), 183–190. doi: 10.1016/s0168-3659(01)00452-7

This paper examined the pharmacokinetics of nicotine in rats following an intravenous administration of nicotine base and nicotine hydrogen tartrate salt. Here the researchers looked at mean residence time (how long the drug stays in the body), system clearance and terminal plasma half life. It should be noted that this study looked at nicotine's traits in Sprague-Dawley rats. First the researchers determined the apparent partition coefficient of nicotine (lipicity, which is the ability of a molecule to cross membrane). Next the researchers examined particle size and determined the plasma binding rate of nicotine. After this, they then examined the distribution of nicotine in the rat blood cells. It should be noted that all this was studied in rats, by performing a surgery on the rats (left femoral artery) that would allow researchers to take blood samples at varying times. Also, the dose for the rats was 1ml/kg, which is consistent with our previous injections.

The results found that the APC for NB was larger and remained the same under varying concentrations while the APC for NS was found to be the same value but decreased over time. For the particle size, researchers looked at the respective nicotine micelles diameters in water, they however could not conclude if nicotine behaved like this in the bloodstream. The binding and distribution between NS and NB was found to be the same. It was found that for NS the alpha half life was 9.0+/- 5.6 min and the beta half life was 51.4+/- 12.2 min.

Topic: Pharmacokinetics of Nicotine
Summary: Age affects metabolic rates and half-life of nicotine

Craig, E. L., Zhao, B., Cui, J. Z., Novalen, M., Miksys, S., & Tyndale, R. F. (2014). Nicotine Pharmacokinetics in Rats Is Altered as a Function of Age, Impacting the Interpretation of Animal Model Data. *Drug Metabolism and Disposition*, *42*(9), 1447–1455. doi: 10.1124/dmd.114.058719

This study aimed to look at pharmacokinetic differences of nicotine and its correlated metabolites between adolescent and adult aged rats. In this study, nicotine was administered to early adolescent or adult aged rats. Additionally, only male rats were used. Rats were given either an acute sc at 1.0 mg/kg or i.v 0.2 mg/kg. It should be noted that this study looked at Wistar rats and used liquid chromatography in order to get their results. My main interest in reading this study was the differences of half-life time between the two differently aged rat groups, because this information could be beneficial to my own experiment. Here the researchers found that the half-life of nicotine in early adolescent rats was 62 mins while the half-life of nicotine in AD rats was 74 mins. Additionally, the researchers attributed the differences of half-life of nicotine in these two groups of rats to adsorption, drug distribution, excretion, and metabolism rates being different due to age.

Topic: Nicotine in Humans
Summary: Smokers smoke in order to decrease anxiety

Parrott, A. C. (1999). Does cigarette smoking cause stress? *American Psychologist*, *54*(10), 817–820. doi: 10.1037/0003-066x.54.10.817

This paper aims to discuss the relationship between smoking, and stress levels through utilizing anecdotal evidence given by smokers. At first glance this relationship seems complicated and paradoxical because smokers report that smoking makes them feel less stressed but report feeling more stressed than non-smokers. In fact, smokers state that mood control and feeling relaxed is a main reason for their smoking. Through the use of surveys researchers found that mood control seemed to be a main reason for continued smoking in smokers despite smokers knowing about the adverse effects associated with smoking. It was also found that smokers report a pattern of mood fluctuations which were greater in more dependent cycles. The anecdotal evidence showed that regular smokers had periods of heightened stress between cigarettes and smoking restored their stress levels to normal. The author then proposed a model that nicotine dependence can cause stress although the traditional model is that smoking causes stress.

Topic: Chronic Nicotine Administration
Summary: Chronic nicotine exposure lead to different neural responses to stress

Benwell, M. E. M., & Balfour, D. J. K. (1982). Effects of chronic nicotine administration on the response and adaptation to stress. *Psychopharmacology*, *76*(2), 160–162. doi: 10.1007/bf00435271

This paper looked at the effects of chronic nicotine administration (0.4 mg/kg for 40 days) and its withdrawal on the adrenocortical response to acute and repeated exposure to stress and how it is related to 5-HT levels. This experiment used the open platform model to determine stress. Here they had 2 groups be given nicotine or saline for 39 days. On day 40, half the rats pretreated with nicotine received nicotine while the other half did not receive nicotine. Here the rats were then stressed via 30mins on the open platform. Half the saline rats were stressed, while the other half of the saline rats were put in their cage for a control// baseline group. In the other part of the experiment, there was the same group of injections as above, but some of the rats were then after being injected with their injections were then stressed for 30 mins daily. Only one group was injected with saline then put back in the home cage.

The results of this experiment showed that there was a difference in the relationship between 5-HT and plasma cortisol, which suggests that the groups of rats adapted to repeated stress in different ways.

Topic: Gender Differences with Nicotine
Summary: The experiment found that male and female rats had different response to nicotine

Booze, R., Welch, M., Wood, M., Billings, K., Apple, S., & Mactuts, C. (1999). Behavioral Sensitization Following Repeated Intravenous Nicotine Administration Gender Differences and Gonadal Hormones. *Pharmacology Biochemistry and Behavior*, *64*(4), 827–839. doi: 10.1016/s0091-3057(99)00169-0

The purpose of this study was to examine gender dependent differences in response to nicotine. This was done because they have not been studied extensively in literature. This study explored whether, behavioral sensitization occurred due to IV administration of nicotine, if sensitization was greater in females than males, if sensitization was modulated by gonadectomy, whether intact female rates had normal estrous cytology patterns in response to chronic nicotine administration, and the pharmacokinetics of IV nicotine dosing. In essence all these questions stemmed from how rats' metabolic rates were different due to different levels of hormones and this study aimed to see if any of these ideas were true. For the experiment the researchers look at male, female, castrated, and ovariectomized rats. Nicotine was administered via a dorsally implanted port for chronic IV injections. Rats received either 50 ug/kg of nicotine or saline for 13 days and on the 14th all rats were given 50 ug/kg of nicotine. Additionally, a catheter was put in. To test for sensitization effects, the researchers had locomotor activity test that they would use to score rats on day 1 and day 14. Using the catheter, the researchers could perform pharmacokinetic analysis by retrieving plasma samples and using a GC/MS analysis of said plasma and performed this analysis on day 14. The researchers found that there was an overall significant main effect of gender on acute nicotine exposure with female rats being more active than male rats. Additionally, in chronic nicotine exposure, there was also an effect of gender with female rats being more active than male rats. The researchers also found that acute administration of nicotine had no overall depression in locomotor activity but chronic nicotine exposure lead to increases in this. In general, the study found in regard to the claims laid out above that 1) behavioral sensitization can occur following IV dosing at 50 ug/kg, 2) females may display greater sensitization than males, 3) the dose of nicotine in this study did not alter estrous cyclic activity.

Topic: Time and Nicotine
Summary: There were time-depend effects on whether nicotine was anxiogenic or anxiolytic

Irvine, E. E., Cheeta, S., & File, S. E. (1999). Time-course of changes in the social interaction test of anxiety following acute and chronic administration of nicotine. *Behavioural Pharmacology*, *10*(6), 691–697. doi: 10.1097/00008877-199911000-00016

The purpose of this experiment was to examine the effects of nicotine on anxiety and its relationship with the delay between administration of nicotine and testing. Here the researchers used a 0.1 mg/kg dose of nicotine and used the social interaction test as the paradigm of choice. Additionally, the researchers only used male rats. The experiment was 4-fold. For the first part researchers gave rats one dose of nicotine (or saline) and examined how rats would perform in the social interaction test 5 minutes after nicotine administration. The following doses given to the rats were: 0.05 mg/kg, 0.1 mg/kg, 0.5 mg/kg or saline. For the second part of the experiment, rats were either given 4 days of saline then tested in the social interaction test or were given 0.1 mg/kg of nicotine for 4 days then tested in the social interaction test. For the 3rd part, rats were either given saline then tested in the social interaction test, 0.1 mg/kg of nicotine then tested in the social interaction test, given 01. mg/kg for 7 days then tested in the social interaction test, or given 0.1 mg/kg for 14 days then tested in the social interaction test. The fourth part of the experiment tested rats either 30 minutes, 1 hour and 3 hours, or 30 hours after the first nicotine injection and then 30 minutes following a second injection on day 2 with .1 mg/kg nicotine. Here the researchers found that at 0.1 mg/ kg anxiogenic and anxiolytic effects can be observed in the social interaction test at different times following injections.

The main findings of the experiment were that social interaction decreased 5 minutes and 1 hour after injection at 0.1 mg/kg but was increased 30 minutes after injection. Interestingly, the researchers also found there to be tolerance to these effects after 7 days of nicotine administration.

Topic: Time and Nicotine, Molecular Basis of Nicotine
Summary: 5 minutes after injection nicotine has anxiogenic effects and calcium ions mediate this

Biala, G., & Budzynska, B. (2006). Effects of acute and chronic nicotine on elevated plus maze in mice: Involvement of calcium channels. *Life Sciences*, *79*(1), 81–88. doi: 10.1016/j.lfs.2005.12.043

In general, the experiment aimed to examine how acute and chronic nicotine administration affected anxiety. To do this, the researchers gathered, male mice and put them in the following groups: 0.1 mg/kg of nicotine, 0.5 mg/kg of nicotine or saline. In each group half of the animals were tested 5 minutes after drug injection and the other half were tested 30 minutes after drug injection. Additionally, a second group of animals was given 6 days of 0.1 mg/kg of nicotine and one the 7th day mice were either 0.1 mg/kg of nicotine or saline and then tested 5 minutes after injection. The researchers did this because they wanted to see if an anxiogenic effect caused by 0.1 mg/kg of nicotine after 5 minutes would persist over a longer pretreatment period. Additionally, the researchers tested mice by measuring their response in the elevated plus maze (EPM) test. Here anxiolytic activity was indicated by increases of time spent in open arms while anxiogenic behavior was dictated by decreases in this time.

The researchers found that acute doses of nicotine (0.1 mg/kg and 0.5 mg/kg) resulted in an anxiogenic effect 5 minutes after injection. The researchers also found that 30 minutes after injection, a low dose created an anxiogenic effect while the higher dose did not cause an effect. Additionally, in the second group of mice that were tested after 6 days of nicotine injections, the researchers found that a tolerance developed to the anxiogenic effect of nicotine.

For the second part of the experiment, the researchers investigated whether calcium channel agonists (CCA) could influence the effects of nicotine on anxiety. Mice were treated with either nimodipine (5 and 10 mg/kg), flunarizine (5 and 10 mg/kg), verapamil (5 and 10 mg/kg), diltiazem (5 and 10 mg/kg) or saline 15 minutes prior to 0.1 mg/kg nicotine or saline administration. Then researchers got groups of mice to receive chronic nicotine treatment and pre treated them with either nimodipine (5 and 10 mg/kg), flunarizine (5 and 10 mg/kg), verapamil (10 and 20 mg/kg), diltiazem (10 and 20 mg/kg) or saline for six days. On the 7th day, mice were given 0.1 mg/kg of nicotine and tested in the EPM. It should be noted that all the drugs used were different types of L-type VDCC antagonists.

In the second experiment the researchers found that CCA pretreatment on acute nicotine exposure significantly reversed the anxio-genic like effects of acute nicotine. Additionally CCA pretreatment for chronic exposure to nicotine was found to abolish the anxiolytic effect of chronic nicotine exposure. This data indicates that calcium ion flow is an essential molecular component of the effects of nicotine

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Biala, G., & Budzynska, B. (2006). Effects of acute and chronic nicotine on elevated plus maze in mice: Involvement of calcium channels. *Life Sciences*, *79*(1), 81–88. doi: 10.1016/j.lfs.2005.12.043

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Craig, E. L., Zhao, B., Cui, J. Z., Novalen, M., Miksys, S., & Tyndale, R. F. (2014). Nicotine Pharmacokinetics in Rats Is Altered as a Function of Age, Impacting the Interpretation of Animal Model Data. *Drug Metabolism and Disposition*, *42*(9), 1447–1455. doi: 10.1124/dmd.114.058719

Davis, J. A., James, J., Siegel, S., & Gould, T. (2005). Withdrawal from Chronic Nicotine Administration Impairs Contextual Fear Conditioning in C57BL/6 Mice. *Journal of Neuroscience*, *25*(38), 8708–8713. doi: 10.1523/jneurosci.2853-05.2005

Irvine, E. E., Cheeta, S., & File, S. E. (1999). Time-course of changes in the social interaction test of anxiety following acute and chronic administration of nicotine. *Behavioural Pharmacology*, *10*(6), 691–697. doi: 10.1097/00008877-199911000-00016

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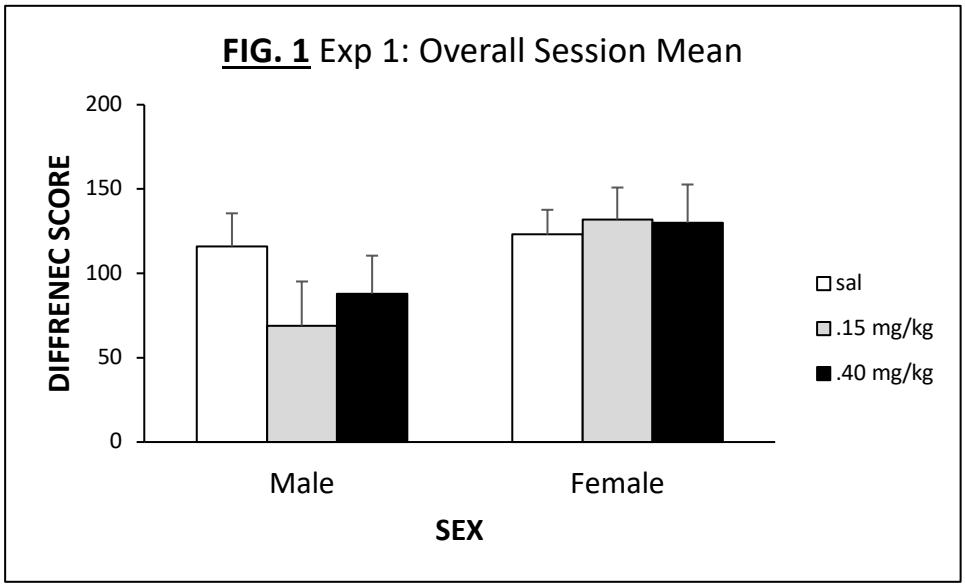


FIG. 1 Exp 1: Overall Session Mean
DIFFRENEC SCORE
200
150
100
50
0
Male
Female
SEX
sal
.15 mg/kg
.40 mg/kg

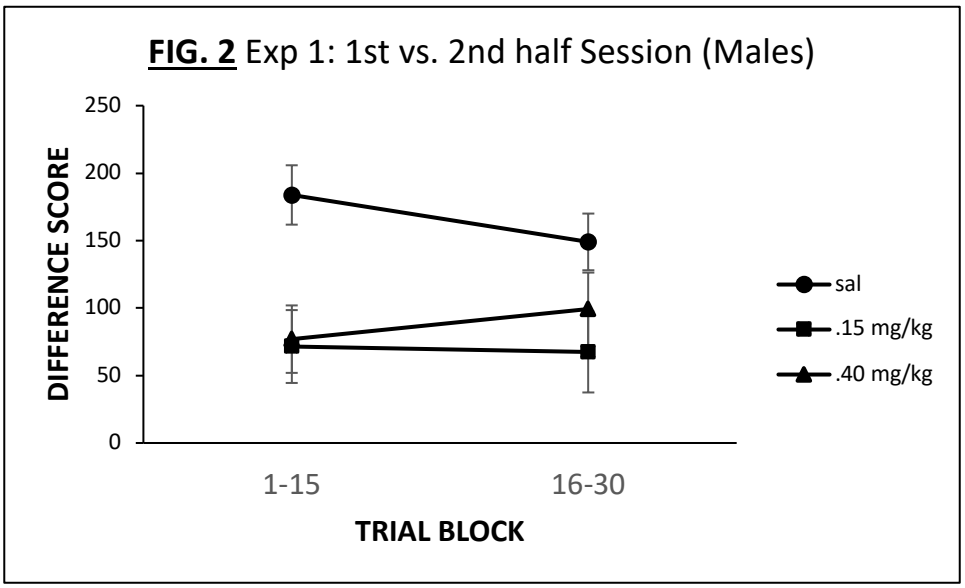


FIG. 2 Exp 1: 1st vs. 2nd half Session (Males)
DIFFERENCE SCORE
250
200
150
100
50
0
1-15
16-30
TRIAL BLOCK
sal
.15 mg/kg
.40 mg/kg

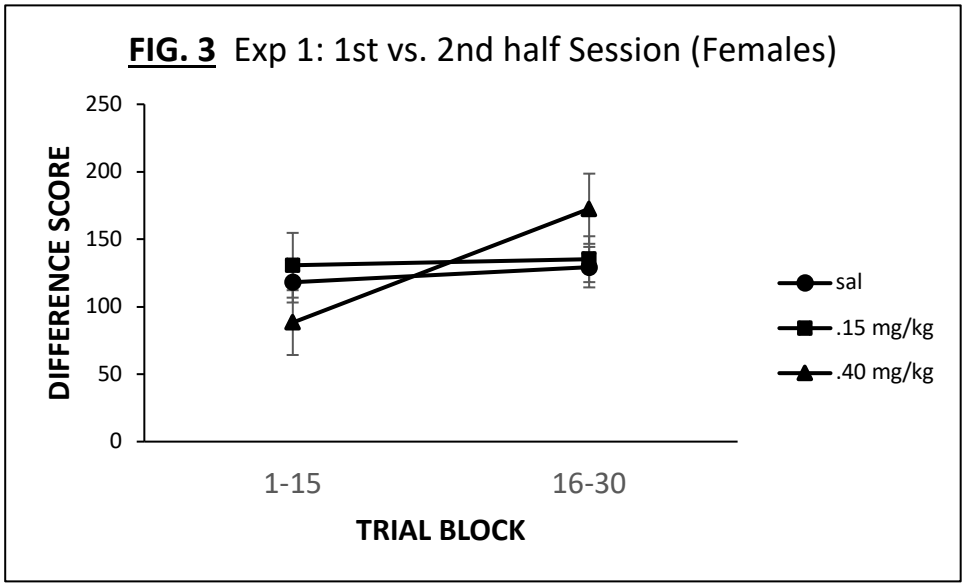


FIG. 3 Exp 1: 1st vs. 2nd half Session (Females)
DIFFERENCE SCORE
250
200
150
100
50
0
1-15
16-30
TRIAL BLOCK
sal
.15 mg/kg
.40 mg/kg

FIG. 4 Exp 1: Overall Session Mean (Test 1 Only)

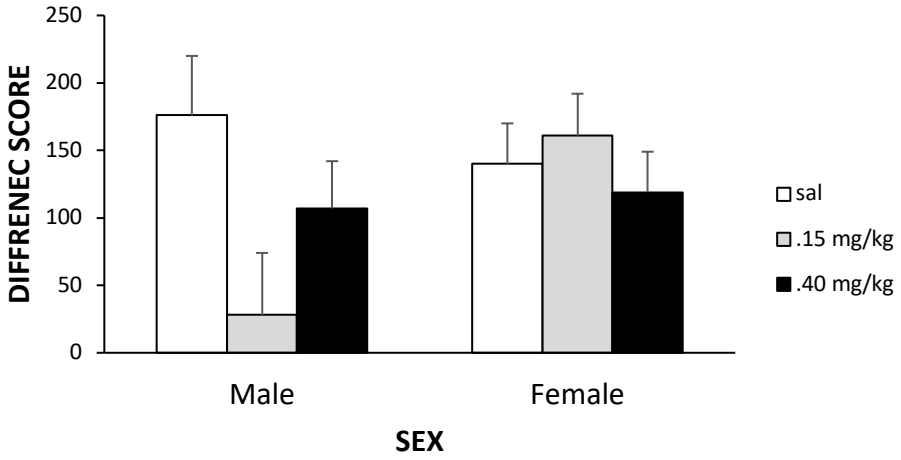


FIG. 5 Exp 1: 1st vs. 2nd half Session (Test 1 Only, Males)

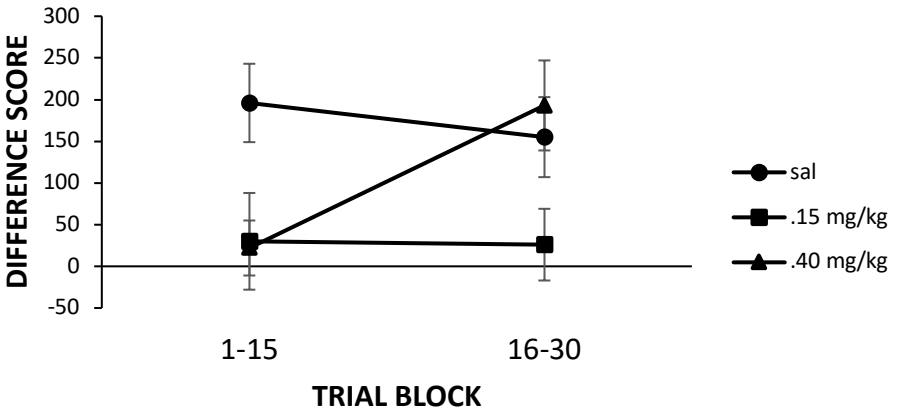
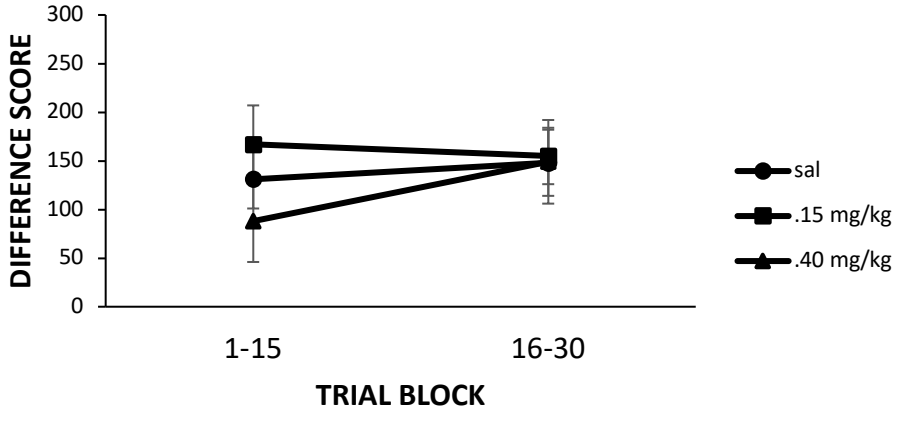


FIG. 6 Exp 1: 1st vs. 2nd half Session (Test 1 Only, Females)



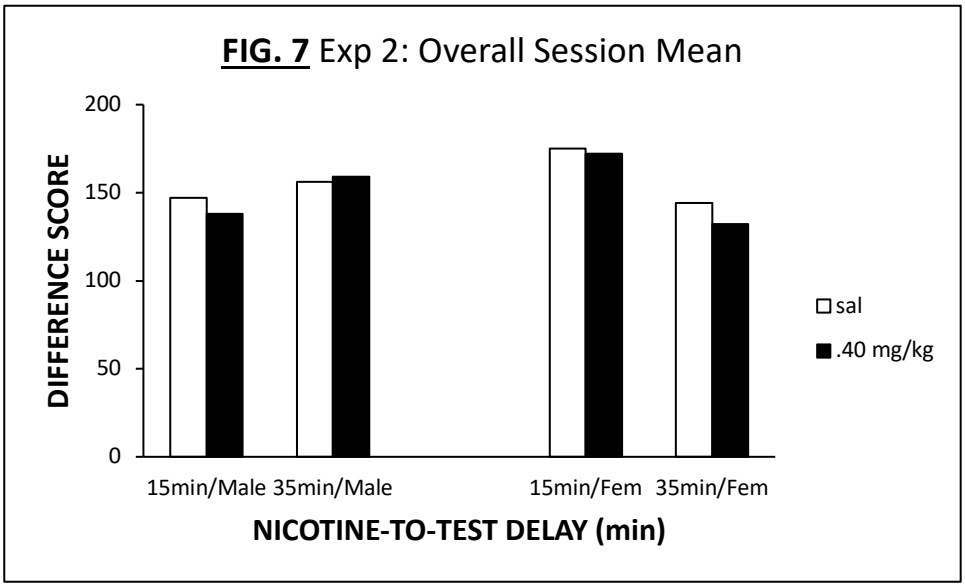


FIG. 7 Exp 2: Overall Session Mean
DIFFERENCE SCORE
200
150
100
50
0
15min/Male
35min/Male
15min/Fem
35min/Fem
NICOTINE-TO-TEST DELAY (min)
sal
.40 mg/kg

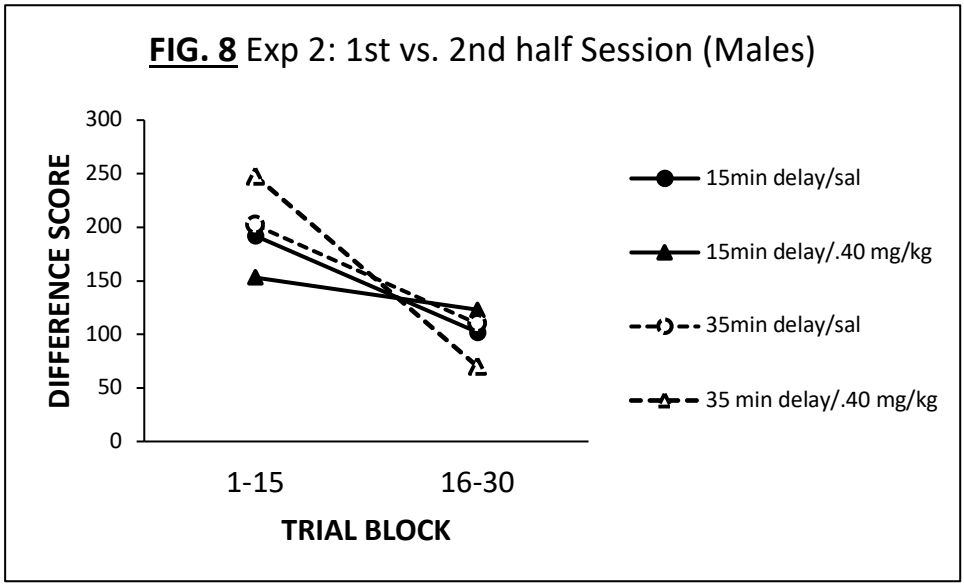


FIG. 8 Exp 2: 1st vs. 2nd half Session (Males)
DIFFERENCE SCORE
300
250
200
150
100
50
0
1-15
16-30
TRIAL BLOCK
15min delay/sal
15min delay/.40 mg/kg
35min delay/sal
35 min delay/.40 mg/kg

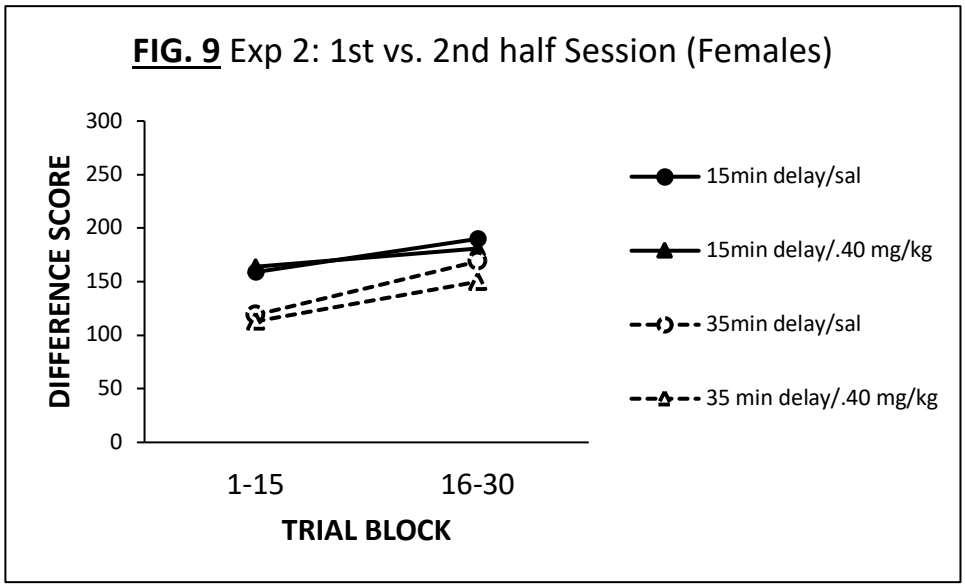


FIG. 9 Exp 2: 1st vs. 2nd half Session (Females)
DIFFERENCE SCORE
300
250
200
150
100
50
0
1-15
16-30
TRIAL BLOCK
15min delay/sal
15min delay/.40 mg/kg
35min delay/sal
35 min delay/.40 mg/kg

FIG. 10 Exp 2: Overall Session Mean (Test 1 Only)

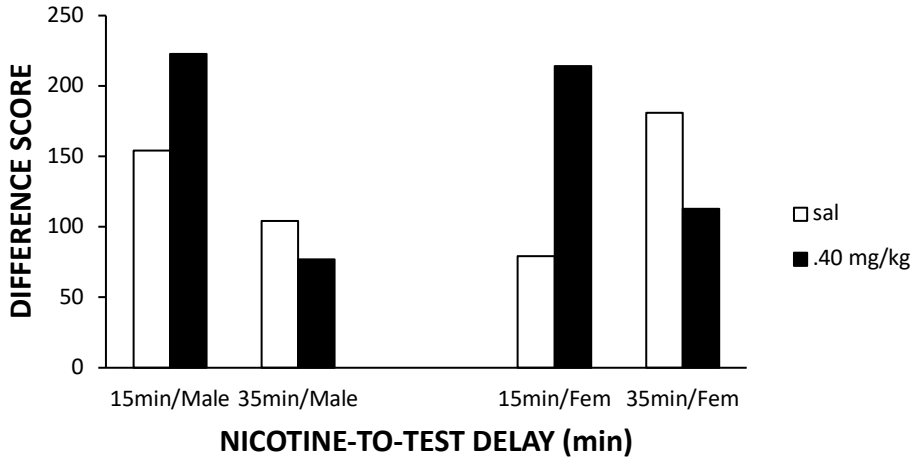


FIG. 11 Exp.1 vs. Exp.2 Overall Session Mean (Test 1 Only, MALES)

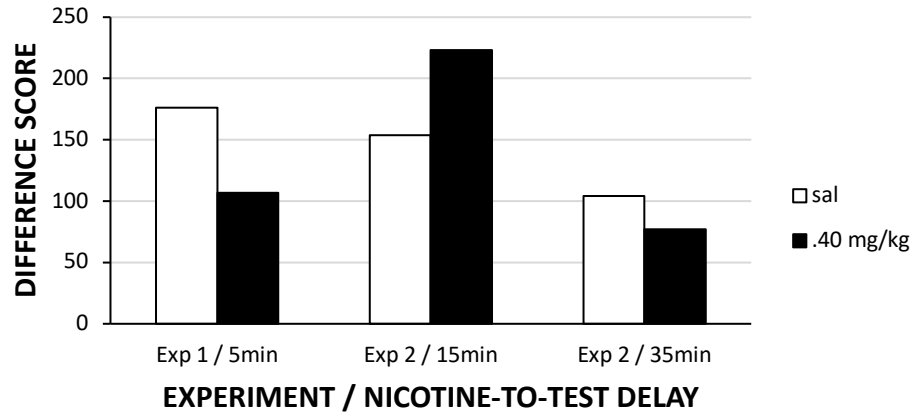


FIG. 12 Exp.1 vs. Exp.2 Overall Session Mean (Test 1 Only, FEMALES)