

SEX DIFFERENCES IN CONDITIONING OF NICOTINE-ASSOCIATED CUES

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Smoking cessation success rates are lower in females. Nicotine replacement therapy (NRT) increases smoking cessation, but successful quit rates remain low. NRT studies demonstrate the complexity of the smoking experience and support the importance of nicotine-associated cues in driving smoking behaviors. The conditioning of nicotine-associated cues represents a potential mechanism through which smoking-related cues may become differentially reinforcing and males and females. AIM: The current study assesses whether nicotine-associated stimuli become conditioned reinforcers and whether this occurs differently across sexes. HYPOTHESIS: A cue associated with nicotine delivery will become conditioned reinforcers and support higher responding on a novel response than a cue not previously associated with nicotine. Conditioning will be greater in females. METHOD: Sprague-Dawley rats (92 m, 92 f) responded on nose pokes for 32 conditioning sessions. Animals in the experimental group (NIC+CS) responded on the active nose poke for a presentation of a 15s white light (CS) accompanied by an infusion of nicotine (NIC). Animals in control groups responded for a NIC (NIC-Only) infusion, the CS (CS-Only) accompanied by a saline infusion, the CS in the presence of yoked NIC (YN+CS), or no reinforcer (Activity-Only). After conditioning, nose pokes were replaced with levers. All animals responded for the CS for 5 sessions. STATISTICAL ANALYSES: Group and sex Differences in the number of reinforcers earned were assessed using pairwise ANOVA comparisons. Findings with $p < 0.05$ were considered significant.

RESULTS: During conditioning, NIC-Only animals ($F=46.8$, $p<0.001$) and CS-Only animals ($F= 58.0$) earned more reinforcers than Activity-Only animals. YN+CS animals demonstrated enhanced responding for the CS compared to CS-Only animals ($F=60.0$). Adding the CS to NIC delivery increased responding for NIC ($F=35.5$) in both sexes, but this increase was greatest in males ($F=4.0$), suggesting greater conditioning in males. During the new response phase, NIC+CS animals earned more CS presentations than CS-Only ($F=19.9$) or YN+CS ($F= 8.7$) animals, suggesting the CS became a conditioned reinforcer. No group*sex interactions were revealed, however power to detect this interaction was low. CONCLUSIONS: Cues associated with nicotine delivery become conditioned reinforcers. Conditioning effects are greater in males than in females.

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1.0 INTRODUCTION

Smoking has long been known to have deleterious effects on health, including increased risk for cardiovascular disease, lung cancer, and myriad health complications (Centers for Disease Control and Prevention, 2005). Smoking also leads to billions of dollars spent on the treatment of such health complications and related loss of productivity (Peto et al., 1999; Centers for Disease Control and Prevention, 2009). There are over 1 billion smokers estimated in the world (Jha, 2002), and despite known health risks, people continue to initiate smoking behaviors (World Health Organization, 2008). Furthermore, among those already smoking, cessation rates are low and many smokers have difficulty quitting. Benowitz (2009) estimates 80% of smokers who attempt quitting on their own relapse within the first month. Success rates increase with the use of nicotine replacement therapy, but most smokers eventually relapse (Benowitz, 1999).

Not surprisingly, researchers are interested in *why* people continue to smoke. What factor or factors could be so powerful as to override a person's knowledge of potential smoking risks and lead the smoker to maintain maladaptive smoking behaviors? Smoking is a complex situation in which the environment, genetic factors, personality factors, and experience of the smoker combine to influence future smoking. The act of smoking allows a person to self-administer nicotine, which is an unconditioned primary reinforcer (Meisch & Lemaire, 1993). The environment in which smoking occurs consists of several cues that have been associated with smoking (i.e. the setting, people in the setting, the sight and smell of smoke) and consistently

predict the delivery of nicotine. In Pavlovian terms (Pavlov, 1927), nicotine acts as an unconditioned stimulus (US). The delivery of nicotine results in an increase of reinforcement. Before being paired with nicotine, cues such as the sight and smell of smoke are not reinforcing. However, these cues are consistently available immediately prior to nicotine delivery and become conditioned stimuli (CS). Through repeated CS-US pairings, presentation of the CS predicts presentation of the US, and results in a conditioned response (CR) similar to the unconditioned response (Rescorla, 1967). In the case of smoking, non-nicotine cues, such as the sight of smoke, predict both nicotine delivery and the onset of nicotine-associated reinforcement. To the extent non-nicotine cues predict reinforcement, cues themselves may become conditioned reinforcers and may help maintain smoking behaviors.

Researchers are also interested in factors that may differentially affect subpopulations of smokers. For instance, lower cessation rates indicate women have more difficulty quitting smoking than men (Scharf & Shiffman, 2004). Females are also less responsive to nicotine replacement therapy (Perkins et al., 1999), suggesting males are more sensitive to the primary reinforcing effects of nicotine. Conversely, female drug-taking behaviors may be more strongly influenced by smoking-related cues (i.e. sight and smell of smoke, holding a cigarette, etc.) and females may benefit more from treatments intended to diminish responses to smoking cues, such as cue-exposure therapy (Perkins, 2001).

1.1 RAT MODEL OF NICOTINE SELF-ADMINISTRATION

Animal models of drug self-administration are used to investigate various features of drug-taking behaviors. Nicotine self-administration models are based on the premise that nicotine acts

as a primary reinforcer and will increase responding if nicotine delivery is response-contingent (Meisch & Lemaire, 1993). Nicotine is self-administered by several species, including rats. Self-administration depends on both nicotine dose and reinforcement schedule, and is extinguished in the absence of nicotine (Corrigall & Coen, 1989). Rat models of self-administration are not unique to nicotine. In fact, self-administration models have been used to assess the effects of such drugs as alcohol (Adams, 1995; Juarez and Barrios de Tomasi, 1999; Roberts et al., 1998), cannabinoids (Braida et al., 2004; Fattore et al., 2001), cocaine (Lynch & Carroll, 1999; Carroll et al., 2004), and heroin (Lynch & Carroll, 1999; Stewart et al., 1992).

Animal models allow researchers to isolate both drug-seeking and drug-taking experiences from the external social and cultural factors that complicate clinical research (Fattore, Fadda, & Fratta, 2009) and also non-drug stimuli and nicotine delivery. Furthermore, animal models allow researchers to examine the influence of cues on nicotine self-administration in naïve animals, controlling for previous exposure to nicotine. Animal models allow researchers the distinct advantage of beginning with nicotine-naïve subjects and a neutral cue, manipulating the relationship between that cue and nicotine exposure, and assessing changes in the reinforcing value of the cue over time. Dissimilar to human studies, research can be performed in isolation (unaffected by exposure to numerous other variables) and with fewer ethical and practical obstacles, in a nicotine-naïve population.

1.2 CONDITIONING

Classical conditioning is predicated on the consistent pairing of two events, the presentation of a conditioned stimulus (CS) immediately prior to the presentation of an unconditioned

stimulus (US; Pavlov, 1927). The unconditioned stimulus (nicotine) has an effect, regardless of the presence or absence of the conditioned stimulus (non-nicotine cues). Presentation of the CS before it has been paired with the US will not have an effect comparable to the effect of the US. Greater consistency of CS-US pairings will lead to CS presentations having greater predictive value of a US presentation (Rescorla, 1967). As this association or predictive value increases, presentation of the CS alone begins to elicit a response similar to the response elicited by the presentation of the US.

In the case of smoking, people will smoke to obtain nicotine. Non-nicotine cues might have little or no reinforcing value at the outset of smoking. The taste of the cigarette or sight and smell of cigarette smoke might not be initially reinforcing and may even be aversive or unpleasant. However, non-nicotine cues are consistently available prior to the delivery of nicotine. The smoke, taste, and feel of the cigarette (as well as other cues), predict the onset of nicotine delivery. These cues become conditioned reinforcers and support behavior. Beyond the delivery of nicotine, sensorimotor smoking cues, such as the taste of smoke and the scratch of smoke in the throat have been related to smoking satisfaction (Rose, 1988) and identified as potential conditioned reinforcers (Stolerman et al., 1973). Indeed, one study found smoking denicotinized cigarettes decreased craving for nicotine, while the administration of intra-nasal nicotine did not (Fertig, Pomerleau, & Sanders, 1986). More recently, participants wearing a placebo patch and smoking denicotinized cigarettes smoked the same number of cigarettes as participants who were smoking higher nicotine yield cigarettes (Donny and Jones, 2009).

Nicotine association has also been tested in animals. One model of assessing nicotine conditioning is the conditioned place preference (CPP) model (LeFoll & Goldberg, 2005). Animals are allowed to choose between spending time in one of two chambers. One chamber is

associated with the delivery of nicotine, the other with the delivery of saline. Tested after several exposure trials, animals should spend more time in the nicotine-associated chamber, as compared to the saline chamber, even in the absence of nicotine. Nicotine has been shown to induce conditioned place preference across a wide range of doses, in both biased and unbiased procedures (for a review, see Le Foll and Goldberg, 2005).

Nicotine-associated cues have, however, been demonstrated to drive nicotine-seeking behavior in self-administration models. Rats trained to self-administer nicotine with a concurrent light/tone cue continued to respond for the cue after nicotine was removed from the response contingency, an effect that persisted over time (Cohen et al., 2005). Results from this study are difficult to interpret, as the initial reinforcing value of the cue (prior to association with nicotine) was not tested and adequate control groups to test for other pharmacological influences of nicotine were not included. Additionally, high responding during the testing phase may have been an artifact of responding on the same operant through training (i.e. an example of habit-learning).

In order to address these issues, Palmatier et al. (2007) used an acquisition of a new response paradigm (Mackintosh, 1974) to show that a neutral cue can be established as conditioned reinforcers in male rats if the cue is repeatedly paired with the delivery of a nicotine infusion. During conditioning trials, animals were placed in one of three groups. Animals in the NIC+CS group nose poked for response-contingent presentations of a 15-second cue light that was accompanied by a nicotine infusion. Animals in the CS-Only group nose poked for response-contingent presentations of the 15-second cue light that were accompanied by a saline infusion. Animals in the Unpaired group received cue presentations and nicotine infusions that were yoked to animals in the NIC+CS group, but nicotine infusions and cue presentations were not allowed

to overlap or occur within 70 seconds of each other. Animals in this group were not allowed to perform the nose poking behavior. It was hypothesized the cue would become more reinforcing when it had been paired with nicotine delivery. Consequently, responding for the cue alone was expected to be highest in animals that had received the cue paired with a nicotine infusion. During the acquisition of a new response phase, animals responded on the active lever to earn presentations of the cue. Animals trained with paired nicotine and cue presentations responded more for the cue, reflecting the ability of the cue to become a conditioned reinforcer. Animals trained in the CS-Only and Unpaired groups responded significantly less for the cue, indicating the cue was less reinforcing when it was not associated with nicotine. A follow-up study revealed the conditioning effects of nicotine are influenced by the strength of the primary reinforcer (Palmatier, 2008). Animals conditioned with higher nicotine doses showed greater break points (i.e. worked harder) on a progressive ratio (PR) task, indicating increased motivation to obtain the conditioned stimulus. Though the previous examples deal with the conditioning effects of nicotine in self-administration paradigms, it is important to note that nicotine can exert conditioning effects via other routes of drug administration.

1.2.1 Reinforcement enhancing effects of nicotine

While previous models of nicotine self-administration have focused on the primary reinforcing actions of nicotine, recent work has shown that nicotine has more than one action (Donny et al., 2003; Palmatier et al., 2006). Self-administration models have repeatedly shown nicotine to be a primary reinforcer (able to maintain contingent responding) and pairing nicotine with cues elevates levels of responding. However, it has been unclear whether the increase in responding was due to responding for nicotine or responding to obtain a cue presentation. Since

the cue and nicotine are *both* obtained by performing on a single operant, it is impossible to determine whether the presence of the cue leads to higher responding for nicotine. In order to ascribe increased responding to conditioning, it must be clear that the change in responding is due to the *pairing* of nicotine and the cue, not simply a result of other pharmacological effects of nicotine. If increased responding for the cue was simply due to a pharmacological effect of nicotine, responding should also increase when nicotine is delivered non-contingently. If increased responding is due to a conditioning effect, responding should only increase when nicotine and the cue are delivery simultaneously and contingent upon behavior.

Donny et al. (2003) addressed this issue and showed increased responding for a reinforcing visual stimulus (VS) in the presence of nicotine, whether delivered contingently or non-contingently. Animals were separated into 6 groups: Contingent NIC+VS, Contingent NIC +No VS, Contingent Saline + VS, and the three non-contingent (yoked) counterparts to these groups. The inclusion of the non-contingent groups in this study allowed researchers to differentiate between increased VS reinforcement as a function of association and increased reinforcement as a function of the non-associative effects of nicotine. Animals in the Contingent Saline + No VS, Contingent NIC + No VS, and Non-Contingent NIC + No VS all responded at low levels. Animals in the Contingent Saline + VS showed greater responding than animals in the Contingent Saline + NO VS condition, indicating the VS was reinforcing. Contingent NIC + VS animals and Non-contingent NIC +VS animals responded similarly and more than animals in the Contingent Saline + VS condition. Additional studies also showed the nicotine-driven increase in responding for the VS could be immediately reduced to Saline + VS levels by substituting saline for nicotine. Responding was immediately reinstated when saline was replaced with nicotine. Palmatier et al. (2006) extended these findings using a two-lever paradigm which

allowed animals to respond on one operant for a nicotine reinforcer and another operant for the VS. Additional animals responded on an active lever for either a nicotine infusion (Nic-Only), a VS presentation accompanied by a saline infusion (VS-Only), or a VS presentation accompanied by a nicotine infusion (NIC+VS). Animals in the two-lever condition responded for nicotine at levels similar to Nic-Only animals. Animals in the two-lever condition earned more VS responses than animals in the VS-Only condition. In fact, two-lever animals earned VS presentations comparable to animals in responding on one lever to earn simultaneous nicotine infusions and VS presentations. These findings indicate that increased responding for the VS when the VS accompanies a nicotine infusion may not be a function of conditioning, but rather a result of the reinforcement enhancing effects of nicotine. Consequently, this study underscores the need to isolate the conditioning effects and reinforcement enhancing effects of nicotine.

Thought it is important to study the conditioning nicotine-associated stimuli and the reinforcement enhancing effects of nicotine separately, it is important to remember the study of each phenomenon informs the study of the other. In order to ascribe increased responding to conditioning, it must be clear that the change in responding is due to the *pairing* of nicotine and is not a result of other pharmacological effects of nicotine. Conditioning mechanisms explain how a neutral cue can become reinforcing when paired with nicotine. Once the cues are reinforcing, their reinforcing value is enhanced in the presence of nicotine (Palmatier, 2007). Reinforcement enhancement mechanisms explain how the reinforcers become more reinforcing in the presence of nicotine. Taken in tandem, these processes define a pathway through which nicotine-related cues become more reinforcing over time, how the presence of nicotine alters their reinforcing value, and how these cues influence smoking behaviors. This highlights the

importance of understanding the mechanisms through which smoking cues become conditioned reinforcers.

1.2.2 Sex differences in the importance of non-nicotine cues

Evidence for sex differences in the importance of cues also exists. Perkins et al. (2001) reported that females showed changes in subjective and reinforcing self-reports of smoking, varying by whether or not subjects were able to experience external smoking cues. Females whose visual and olfactory experience of smoking was blocked expressed lower ratings of satisfaction and hedonic responses, as well as lower levels of reinforcement (via administration of cigarette puffs). This was not the case with males, suggesting cues are more important in the maintenance of smoking behaviors in women compared to men.

In a follow-up study (2002), the subjective and reinforcing effects of nicotine were less affected by nicotine dose (cigarettes) in women compared to men. Participants abstained from smoking overnight, and then performed a progressive ratio task to earn cigarette puffs. Smokers earned puffs from either a “moderate” or “low-nicotine” cigarette, one dose on each of two days, presented in a balanced order. After the progressive ratio task, participants rated subjective measurements of reinforcement such as satisfaction, liking, and how much they would be willing to pay for a similar cigarette. In both sexes, a dose effect was present in measures of reinforcement. Participants reported greater reinforcement after smoking the “moderate” nicotine cigarettes, as compared the “low” nicotine cigarettes. However, this difference was significantly smaller in females than in males. That is, for women, nicotine dose was less important than other influences driving reinforcement from smoking. In yet another study (Perkins, 2006), the effects of dosing instructions on the rewarding effects of smoking were

assessed. In women, reinforcement and self-reports of reward varied depending on what subjects were *told* cigarettes contained. Women who were told they were smoking cigarettes containing nicotine reported higher levels of reinforcement after smoking than women who were told they were smoking denicotinized cigarettes. In males, reinforcement and reward varied as a function of what cigarettes *actually* contained. Again, this suggests that non-nicotine information influences smoking more for females than for males.

Preclinical studies have also assessed potential sex-differences in nicotine-association. Male rats acquire nicotine-conditioned place preferences, while female rats do not (Yararbus et al., 2010). However, studies have shown both male and female rats will acquire nicotine self-administration (Donny, 2000) and have implicated sex differences in the importance of cues for nicotine self-administration (Chaudhri et al., 2005). In a rat model, both males and females were allowed to establish stable nicotine self-administration (in the absence of cues) across several doses (0.03, 0.06, and 0.15 mg/kg/infusion). No sex differences in self-administration were revealed at the lowest nicotine dose. However, females self-administered more than males at both the 0.06 and 0.15 mg/kg/infusion doses. Following stabilization, animals continued to self-administer nicotine; however, responding now resulted in delivery of both a nicotine infusion and a cue presentation. The cue used in the study was a weakly-reinforcing visual stimulus (VS): a one-second light presentation followed by a 1-minute house light off. The presence of the VS increased responding in both males and females. Responding increased more for females in all dose conditions, but this difference was significant only for 0.03 and 0.06 mg/kg/infusion doses. Subsequent removal of the VS resulted in decreased responding and sex differences were no longer evident. This study suggests the presence of cues increases nicotine self-administration, and this effect is more robust in females than in males. It is important to note,

when tested alone and in animals with no history of nicotine self-administration, the VS was differentially reinforcing. Females responded more for the VS than males. This suggests that the presence of nicotine may enhance responding more for a cue that is more reinforcing. When a nicotine infusion was paired with VS delivery, females self-administered nicotine more than males. This study establishes the need to understand how cues (such as the VS) may become differentially reinforcing in males and females and how nicotine further affects the reinforcing value of cues. It does not, however, disentangle conditioning effects and the reinforcement enhancement effects of nicotine. In order to disentangle these effects, it is necessary to address the conditioning effects of nicotine in a more stringent manner. The effects of conditioning must be assessed not through between-group differences in responding (some of which take place in the presence of nicotine), but also by testing the reinforcing value of the cue in the absence of nicotine, by its ability to support responding on a new response.

1.2.3 Current study

Previous research has shown nicotine self-administration in both males and females and has indicated the presence of cues may influence nicotine self-administration. The literature fails to adequately disentangle the conditioning effects of nicotine from the reinforcement enhancing effects of nicotine. The current study seeks to provide a more stringent test of the conditioning effects of nicotine by using an acquisition of a new response paradigm. In this paradigm, animals self-administer conditioning contingencies for several days, after which all animals respond on a novel operant for the conditioned stimulus. This design allows for testing the reinforcing value of the cue, while eliminating the potential confound of testing for responding

for the cue in the presence of nicotine. Furthermore, the current study examines whether the conditioning effects of nicotine varies across sexes.

The current study consists of three, smaller studies. Study 1 examines the reinforcing properties of a 15-second white cue light. Study 2 and Study 3 use an acquisition of a new response paradigm to investigate the conditioning of non-nicotine stimuli by experimentally manipulating the association between an unconditioned stimulus (nicotine) and a conditioned stimulus (15s white cue light from Study 1) in both males and females.

1.2.4 Hypotheses

A 15s white cue light will be equally reinforcing for males and females. Nicotine will act as a primary reinforcer. Animals responding on an operant for contingent nicotine will respond more than animals who respond on the operant without reinforcement. This effect will be consistent across sexes. A neutral cue that is repeatedly paired with nicotine will take on the properties of nicotine and become a conditioned reinforcer. Animals earning simultaneous nicotine infusions and cue presentations during the conditioning phase will respond more for the cue during acquisition of a new response, as compared to animals not earning nicotine infusions and cue presentations simultaneously. This difference is expected to be exaggerated in females, reflecting greater conditioning of nicotine-associated cues.

2.0 METHODS AND RESULTS

2.1 STUDY 1: ESTABLISHMENT OF A NEUTRAL CUE

2.1.1 Subjects

Subjects were sixty Sprague-Dawley rats (Harlan farms), aged approximately 3 months and weighing 175 to 225 grams at the start of the experiment. Upon arrival, rats habituated to their home cages for at least one week. Rats were housed individually in a temperature controlled environment under a 12 hour reversed light/dark cycle (lights off at 6 a.m.). Animals were fed once daily in their home cages following experimental sessions. Daily food intake was controlled at 20 grams per day, and rats were allowed unlimited access to water in their home cages. This feeding pattern results in a gradual weight gain of approximately 15 g/week (Donny et al. 1995). Sessions were conducted between the hours of 6 a.m. and 6 p.m.

2.1.2 Apparatus

Behavioral testing was carried out in eighteen 25x31x28 operant test chambers (MED Associates, Inc., St. Albans, Vermont, USA), each enclosed in a sound-attenuated cubicle. On the right wall of the chamber, two nose pokes (one on the left, one on the right) were available 3 cm above the floor. A white stimulus light was located 5 cm above each nose poke.

2.1.3 Design

Sprague-Dawley rats (30 male, 30 female) were used to test the influence of unconditioned reinforcing properties of the proposed visual stimulus, including differential responding between sexes. The hypothesis being tested in study two considers whether nicotine increases the reinforcing value of a visual stimulus through conditioning. Assessing differences between sexes in the conditioning effects of nicotine on nicotine-associated stimuli between sexes requires the use of a cue that is equally non-reinforcing for males and females prior to conditioning.

A small set of animals was used to test between-sex differences in responding for a 15-second white stimulus light. Both males (n=15) and females (n=15) responded for presentations of the stimulus alone (STIM). An additional 15 males and 15 females were assigned to a no-stimulus condition (NO STIM) in which responding had no consequence; see Table 1.

The procedure for establishing the stimulus used a within-subject design similar to the procedure used to assess conditioning. During the first phase of the experiment animals nose poked for cue presentations based on the contingencies stated above. Two nose pokes were available: one active, and one inactive. The location (left or right) of the active nose poke was randomly assigned and balanced across groups. For STIM animals, active nose poke responses were reinforced on a fixed-ratio (FR1) schedule with a one-minute timeout period. Responding on the active nose poke resulted in the onset of the stimulus light for 15 sec; the timeout period began when the stimulus light was illuminated and ended 45 sec after the stimulus light was extinguished. Responses on the active nose poke during the time out period and responses on the inactive lever were recorded without consequence. Animals in the NO STIM (15 males, 15

females) condition were treated similarly, but did not receive presentations of the 15 sec stimulus light. All animals nose poked on an FR1 for 10 sessions, until responding stabilized.

Following stable nose poke responding, nose pokes were removed from the operant chambers and replaced with levers. Again, two levers were available: one active and one inactive. Reinforcers continued to be delivered on an FR1 schedule for 10 sessions. Animals in the STIM group responded on the active lever to earn presentations of the 15 sec stimulus light. Animals in the NO STIM group were treated similarly, but did not receive presentations of the 15 sec stimulus light. Time out conditions were identical to the previous phase. In all animals, time out responses and responses on the inactive lever were recorded but had no consequence. After 10 FR1 lever pressing sessions, all animals were switched to an FR2 for 3 sessions. All lever pressing sessions lasted for one hour and occurred 5 days per week.

2.1.4 Statistical analyses

Differences in responding were assessed using a mixed-model ANOVA. Separate analyses were performed for nose poke, lever press FR1, and lever press FR2 phases. In each analysis, day was treated as the within-subject factor; sex and cue condition (STIM or NO STIM) were treated as between-subject factors. Findings with $p < 0.05$ were considered to be significant.

2.1.5 Results: study 1

During the nose poke phase, a significant effect of day was revealed ($F=180.9$, $p < 0.001$), with the number of reinforcers earned decreasing across day. Neither sex ($F=3.6$, $p=0.063$) nor cue condition ($F=0.2$, $p=0.643$) influenced the number of reinforcers earned. No interactions

between variables were revealed. No difference between active and inactive responding was seen during the nose poke phase, consistent with a lack of cue effect.

Day remained a significant predictor of the number of reinforcers earned during the FR1 lever press ($F=44.2$, $p<0.001$). Again, reinforcers earned decreased across day. Cue condition significantly influenced the number of reinforcers earned during FR1 lever press responding ($F=13.3$, $p=0.001$). Animals in the STIM condition earned more reinforcers (8.5 ± 0.556) than animals in the NO STIM condition (5.6 ± 0.556). Sex did not influence reinforcers earned ($F=0.9$, $p=0.335$), nor did any variables interact. Both day ($F=5.5$, $p=0.023$) and cue condition ($F=18.1$, $p<0.001$) continued to influence the number of reinforcers earned during the FR2 lever pressing phase. The direction of effect in both cases was consistent with the direction from earlier phases. Again, sex did not influence the number of reinforcers earned ($F=1.0$, $p=0.315$) and no interaction between variables was detected. During both lever pressing phases, active responding was higher than inactive responding in animals earning cue presentations. This is consistent with STIM animals earning more presentations and suggests at least some reinforcing value of the cue. Cue effects were not influenced by sex, suggesting the cue was equally reinforcing across sexes. Data from all phases are represented in Figure 1.

Ideally, conditioned reinforcing should be assessed using a neutral stimulus. However, it is very difficult to find a completely neutral stimulus. Since no significant sex differences in unconditioned reinforcement were detected, the 15 sec white stimulus light was considered acceptable CS for Study 2 and Study 3.

2.2 STUDY 2: NICOTINE CONDITIONING

2.2.1 Subjects

Male (n=47) and female (n=49) Sprague-Dawley rats (Harlan farms), arrived at approximately 3 months in age and weighed 175 to 225 grams. Upon arrival, animals habituated to home cages for one week. Animals were housed individually in a temperature controlled environment under a 12-hour reversed light/dark cycle (lights off at 6 a.m.). Animals were fed once daily in home cages following experimental sessions. Daily food intake was controlled at 20 grams per day. Rats were allowed unlimited access to water in their home cages. Sessions were conducted between the hours of 6 a.m. and 6 p.m.

2.2.2 Surgery

Following habituation, rats were implanted with right jugular catheters under isoflurane anesthesia, and allowed a minimum of seven days healing time before beginning self-administration. Cannulae were flushed daily with 0.1 ml of sterile saline containing heparin (30 U/ml) and Timentin (66.67 mg/ml) to maintain catheter patency and prevent infection. In addition, rats received streptokinase for the first four days post-surgery. Catheter patency was tested following the final self-administration session by observing loss of the righting reflex following an infusion of 0.01 mg/kg methohexital. Only results from animals that passed the patency test are reported.

2.2.3 Self-administration apparatus

Behavioral testing was carried out in the same testing chambers described above, with one exception. A 1 cm diameter hole in the top of the chamber gave access to a drug-delivery swivel system, which connected to the implanted catheter and allowed nearly unlimited movement throughout the chamber.

2.2.4 Self-administration drug

Nicotine hydrogen tartrate salt (Sigma, St. Louis, MO) was dissolved in 0.9% saline, and the pH of the solution was adjusted to 7.0 (± 0.2) with dilute NaOH. Nicotine infusions were delivered at a volume of 0.1ml/kg/infusion in less than 1 second. The resulting unit infusion dose was 0.03 mg/kg/infusion and was calculated from the base form.

2.2.5 Design

Study 2 assessed potential sex differences in the conditioning of nicotine-related cues. The experiment followed a 2x4 between subjects design, with sex and group as independent variables. Study 2 consisted of two phases: one in which cue conditioning occurred, and one in which the value of the cue was tested.

2.2.6 Behavioral training: conditioning phase

Nose poke responses in the conditioning phase resulted in either a CS presentation accompanied by a saline infusion (CS-Only), a CS presentation in the presence of yoked nicotine (YN+CS), a CS presentation accompanied by a nicotine infusion (NIC+CS), or a nicotine infusion without a CS presentation (NIC-Only). The NIC+CS group was the primary experimental group representing the association between the CS and nicotine. The NIC-Only group served as a control for history of NIC reinforcement. The CS-Only group acted as a control for the history of CS self-administration. Active nose poking in the YN+CS group resulted in a presentation of the 15 sec stimulus light. Simultaneously, YN+CS animals received NIC infusions yoked to an animal in the NIC+CS group. Each time a NIC+CS animal earned an infusion of nicotine, the YN+CS animal also received an infusion of nicotine. Hence, the YN+CS group served as a control for the self-administration of CS presentations in the presence of nicotine. All groups experienced a one-minute time out period beginning with the delivery of the reinforcer. For animals in the YN+CS group, the time out period began at the onset of the cue presentation. For all other groups, time out began with infusion delivery. For animals in all groups, time out responding and responding on the inactive nose poke were recorded without consequence. The location of the active nose poke (left or right) was randomly assigned and balanced across groups. Responding was reinforced on an FR1 schedule for the first 15 sessions. During sessions 16-32, responding was reinforced on an FR2 schedule. Experimental sessions lasted for one hour and occurred 5 days per week.

2.2.7 Acquisition of a novel response: testing the reinforcing value of the cue

An acquisition of a novel response paradigm was used to test whether the CS became a conditioned reinforcer. The number of CS presentations earned reflected the reinforcing value of the CS. Greater CS presentations earned indicated greater reinforcing value of the CS. Nose pokes were removed from the testing chambers and replaced with levers. For animals in all groups, active lever responding resulted in the 15 sec CS and a saline infusion. Sessions lasted for one hour and occurred 5 days per week. For reasons discussed in detail below, after five sessions, the pump was turned off and active lever pressing resulted in the delivery of a CS presentation without the saline infusion. Testing continued under these criteria for five sessions.

Testing the reinforcing value of the CS should rely on responding for the CS alone. Including a SAL infusion with the delivery of the CS carries additional cues that may have also become conditioned reinforcers over time. These conditioned reinforcers may have differential effects across groups. For instance, the sound of the pump has come to predict delivery of NIC in NIC+CS, NIC-Only, and YN+CS animals and delivery of the CS and SAL (no drug) in CS-Only animals. Responding for the CS with a SAL infusion remains an accurate assessment of the value of the CS for CS-Only animals. However, responding for the CS in the presence of a SAL infusion confounds our ability to assess the reinforcing value of the CS in NIC+CS, NIC-Only, and YN+ CS animals. It becomes impossible to differentiate responding for the CS (i.e. STIM light) from responding for the other conditioned reinforcers (i.e. sound associated with a SAL infusion). In order to eliminate the potential confound, the SAL infusion accompanying delivery of the CS was removed after the first 5 days of the acquisition of a new response phase. Since the change was made after 5 days of acquisition and 5 days into the extinction of nicotine responding (in NIC+CS and NIC-Only animals), it was impossible to determine the effect of the

SAL infusion on the acquisition of the new response. Study 3 was designed to test that effect, but having the entire acquisition of a new response phase take place without infusions.

2.3 STUDY 3: NICOTINE CONDITIONING AND THE ROLE OF A SECONDARY REINFORCER

2.3.1 Subjects

Sprague-Dawley rats (59 males, 56 females) aged approximately 3 months and arriving at between 175 and 225 grams were used to assess the role of a secondary reinforcer (i.e. an auditory cue signaling an upcoming infusions) in driving responding for a previously conditioned cue and a secondary reinforcer. Housing and feeding conditions were identical to Study 1.

2.3.2 Surgery and apparatus

Surgery and apparatus conditions were identical to Study 2.

2.3.3 Design

In the acquisition of a novel response phase of Study 2, animals in the NIC-Only group and NIC+CS group did not differ on the number of CS presentations earned. High responding in the NIC-Only group was hypothesized to be due to combined responding for the auditory cue previously associated with NIC delivery and the acquisition of a new response for a novel

stimulus (the CS). Study 2 did not include a control group that would allow for adequate testing of the hypothesis. Therefore, Study 3 was designed to assess potential sex differences in the conditioning of nicotine-related cues under more stringent criteria. The experiment followed a 2x5 between subjects design, with sex and group as independent variables. The conditioning phase of Study 3 was identical to that of Study 2, with the addition of a control group. The Activity-Only group (ACT) was included to illustrate the value of the CS when previous behaviors have not been reinforced. Hence, this group acted as a control for earning a novel cue during the acquisition of a new response phase. Animals in the ACT group (11 male, 11 female) responded freely on both the active and inactive nose pokes during the conditioning phase. Active nose poking in the conditioning phase did not result in the delivery of a reinforcer, but responses were recorded similarly to all other groups. The remaining groups were treated as explained in Study 2. Animals were assigned to one of five groups including the experimental group (NIC+CS) and four control groups: NIC-Only, CS-Only, yoked nicotine with CS (YN+CS), and activity only (ACT).

Again, an acquisition of a novel response paradigm was used to test whether the CS became a conditioned reinforcer. It was hypothesized that the SAL infusion could be acting as a secondary reinforcer in groups which had received NIC infusions during the conditioning phase. If the pump was acting as a conditioned reinforcer, it would be impossible to differentiate responding for the cue from responding for the secondary reinforcing properties of the pump. In Study 3, all animals lever pressed for contingent CS presentations that were *not* accompanied by a saline infusion during acquisition.

2.3.4 Statistical analyses

All data was analyzed using ANOVA. The nicotine conditioning phase represented responding in a traditional self-administration paradigm with the inclusion of sex as a between subjects factor. All analyses included the linear effect of day (experimental session) as a within subjects factor to assess potential sex differences in acquisition. Sex and group were included as between-subjects factors. The dependent variable of interest in all analyses was the number of reinforcers earned, including both the number of infusions and the number of CS presentations earned. With the exception of the additional ACT group, conditions between Study 2 and Study 3 were identical in the conditioning phase. As a result, data from both studies was combined. In order to account for possible differences between studies, study was included as between-subject factor. When group by study interactions were seen, analyses were performed on each study separately. Differences between studies are reported only when group by study interactions resulted in different findings across studies. Absence of the ACT group in Study 2 does not allow for the inclusion of an omnibus test of group differences. However, the inclusion of the omnibus test results in a multiple degree of freedom test with little specificity. As a consequence, only planned, pairwise comparisons with one degree of freedom are reported. Comparisons dealing with animals in the Activity-Only condition only compare groups from Study 3. Because the length of FR1 and FR2 periods was not identical, separate analyses were performed for FR1 and FR2 phases.

Responding during the acquisition of a novel response phase is complex. Animals are acquiring a novel response, which should result in increased behavior. However, some animals are also experiencing extinction of the association between performing a response and delivery of NIC and between earning a CS presentation and the delivery of NIC. To address this

complexity, the number of CS presentations earned was assessed in multiple ways. First, the maximum number of CS presentations earned in one session of the novel response phase was determined for each animal. Second, rolling three-day means of CS presentations earned were calculated. The maximum three-day mean was taken for each animal. This approach was taken in an effort to assess group differences in CS presentations in a manner sensitive to individual differences in rates of extinction. Though acquisition of a novel response phases in each study lasted 10 sessions, only data from the first five sessions were used. This enables data from both studies to be compared and potential differences due to an additional secondary reinforcer (i.e. the sound of the NIC pump) to be assessed. Only data from animals passing the patency test was used in all analyses.

Some animals were removed from the study due to failed catheters. The final number of animals per group assignment is shown in Table 2 (Study 2) and Table 3 (Study 3).

2.3.5 Results: study 2 and study 3

Data from females are represented in Figure 2; data from males are represented in Figure 3.

Nicotine Conditioning: Reinforcers Earned During FRI Conditioning

Results from main comparisons are summarized in Table 4. Both NIC and the CS supported operant behavior when compared to responding in the absence of consequence (Activity-Only condition; NIC-Only infusions: 9.2 ± 0.7 ; CS-Only presentations: 9.4 ± 0.5 ; Activity-Only: 3.4 ± 0.7). NIC became more reinforcing over time, as evidence by a significant group* linear day interaction. Responding was further increased by the combined effects of NIC and the CS. Animals in the NIC+CS group earned more reinforcers (15.3 ± 0.7) than animals in either NIC-Only or CS-Only groups. Group differences between NIC+CS and NIC-Only or CS-Only

animals increased across day, as evidenced by group*linear day effects. Interestingly, high responding for the CS was also observed in the presence of non-contingent NIC; the number of reinforcers earned by animals in the NIC+CS group did not differ significantly from the number of reinforcers earned by animals in the YN+CS group (13.8 ± 0.7) during FR1 responding.

In general, sex did not moderate the effects of NIC alone or the CS alone. However, the effect of adding the CS to contingent NIC (i.e. NIC+CS vs. NIC-Only) was greater in males than in females, as evidenced by a significant sex*group interaction. Sex*group interactions were not observed when NIC+CS animals were compared to CS-Only animals or when YN+CS animals were compared to either NIC+CS or CS-Only animals. Activity-Only animals did not differentiate between active and inactive nose pokes. Animals in all other groups responded more on the active lever as compared to the inactive lever.

Nicotine Conditioning: Reinforcers Earned during FR2 Conditioning

Results from main comparisons are summarized in Table 5. Both NIC and the CS continued to support operant behavior relative to behaviors performed with no consequence. NIC-Only animals earned more infusions (9.6 ± 0.9) and CS-Only animals earned more CS presentations (6.1 ± 0.4) than Activity-Only animals (1.1 ± 0.8). Group differences due to the reinforcing properties of NIC increased across day, as reflected by a significant group*linear day interaction. Group differences related to the reinforcing value of the CS remained stable across FR2 responding. Similar to during FR1, responding was increased by combining the effects of NIC and the CS. Animals in the NIC+CS group earned more reinforcers (18.2 ± 0.8) than NIC-Only or CS-Only animals. Again non-contingent NIC increased the responding for the CS, with YN+CS animals earning 13.3 ± 0.6 CS presentations.

In general, sex did not moderate the effects of NIC or the CS alone. Similarly to FR1 responding, adding to the effects of the CS to responding for contingent nicotine increased the number of reinforcers earned to a greater degree in males as compared to females. In the NIC-Only condition, males earned an average of 6.8 reinforcers. Adding the CS to contingent NIC increased the number of reinforcers earned to 19.4. Females in the NIC-Only condition earned 10.8 reinforcers compared to 17.0 reinforcers earned by females in the NIC+CS condition. Sex*group interactions were not detected when NIC+CS animals were compared to CS-Only animals or when YN+CS animals were compared to either NIC+CS or CS-Only animals. Activity-Only animals did not differentiate between active and inactive nose pokes. Animals in all other groups responded on the active lever more than on the inactive lever. In order to determine if sex differences at the end of the study were masked by including each day in the analyses, a separate set of analyses were performed using mean reinforcers earned across the last three days of self-administration. As responding can vary from day to day, the three-day mean was chosen to be a more stable measure of reinforcers earned. Using the same comparisons stated above, no additional sex*group interactions were revealed.

Acquisition of a Novel Response

Figure 3 illustrates the number of CS presentations earned by females across each day of the acquisition of a new response phase. Figure 5 illustrates the number of CS presentations earned by males across each day of the acquisition of a new response phase. Both figures represent raw data. Analysis performed using raw data yielded similar results to analyses reported. As described earlier, acquisition of a new response analyses focus on the maximum number of CS presentations earned (Figure 6) and the maximum rolling average. Since maximum number of

CS presentations and maximum rolling average analyses yielded similar results, only maximum number of CS presentations earned is represented graphically.

Maximum CS Presentations Earned

The maximum number of CS presentations earned did not differ between YN+CS (10.4 ± 0.9) and CS-Only animals (8.8 ± 0.8 ; $F=1.8$, $p=0.1$), suggesting the reinforcing value of the CS was not altered by responding for the CS in the presence of non-contingent NIC. NIC+CS animals earned more CS presentations (14.3 ± 0.9) than CS-Only animals ($F=19.9$, $p<0.001$) and YN+CS animals ($F=8.7$, $p=0.004$), suggesting the value of the CS was increased when NIC was delivered contingent upon behavior and simultaneous with CS delivery.

The maximum number of CS presentations earned was significantly lower in CS-Only animals as compared to both NIC-Only (16.9 ± 1.0 ; $F=35.2$, $p<0.001$) and Activity-Only (12.0 ± 0.9 ; $F=8.8$, $p=0.005$) animals. Maximum CS presentations did not differ between NIC+CS animals and either NIC-Only animals (16.9 ± 1.0 ; $F=2.8$, $p=0.097$) or Activity-Only animals (12.045 ± 0.906 ; $F=0.7$, $p=0.7$). Taken together, these findings suggest combining CS and NIC delivery contingent upon behavior results in a cue with reinforcing value similar to the reinforcing value a novel cue that is obtained after previous, repeated exposure to nicotine. Sex differences in the maximum number of CS presentations were not revealed in any comparison.

Maximum Rolling Average of CS Presentations Earned

Comparisons assessing group differences in the maximum rolling average of CS presentations earned revealed findings consistent with analyses using the maximum number of CS presentations earned. YN+CS (8.3 ± 0.8) and CS-Only (6.7 ± 0.6) animals did not differ on the number of CS presentations earned ($F=3.1$, $p=0.08$), suggesting non-contingent NIC did not significantly alter the reinforcing value of the CS. Contingent NIC paired with CS increased the

reinforcing value of the CS. NIC+CS animals earned more CS presentations (11.8 ± 0.7) than both CS-Only animals ($F=26.3$, $p<0.001$) and YN+CS animals ($F=9.7$, $p=0.003$).

Again, CS-Only animals earned fewer CS presentations than either NIC-Only ($F=42.3$, $p<0.001$) or Activity-Only (6.6 ± 0.7 ; $F=9.9$, $p=0.003$) animals. The maximum number of CS presentations earned by animals in the NIC+CS group was similar to both animals in the NIC-Only ($F=2.5$, $p=0.111$) and Activity-Only ($F=0.3$, $p=0.5$) groups. Again, combining the effects of CS and contingent NIC prevents the reinforcing value of the cue from degradation over time. Sex differences were not revealed in any comparison.

3.0 DISCUSSION

As expected, nicotine acted as a primary reinforcer in both males and females. Animals responding for contingent nicotine responded more than animals responding in the absence of a reinforcer. This finding is consistent with the literature (Meisch & Lemaire, 1993; Donny et al. 2000). The 15s white cue light was also reinforcing; across multiple studies, animals responding for contingent cue presentations responded more than animals responding in the absence of a reinforcer. No sex differences in nicotine or cue self-administration were revealed, a result consistent with Chaudhri et al. (2005).

Contingent nicotine increased responding for the cue, a difference that was greater in males than in females. This finding suggests males may be more sensitive than females to the conditioning effects of nicotine. Consistent with the work of Donny et al. (2003), the presence of non-contingent nicotine also increased responding for the cue, confirming the reinforcing value of the cue and indicating enhancement of the reinforcing value of the cue in the presence of nicotine. Enhancement was detected in both males and females; no sex differences in enhancement were detected. This finding is not consistent with previous findings (Chaudhri et al., 2005) indicating greater enhancement in females. In that study, researchers used a moderately reinforcing stimulus; the current study utilized a cue that was intentionally only weakly reinforcing. It is possible that nicotine enhances cues differentially between sexes as a function of the reinforcing value of the cue. In males, nicotine may enhance the value of weak

reinforcers. In females, however, nicotine may only enhance the value of stronger reinforcers. More research needs to be performed to assess which factors (i.e. strength of the reinforcer) influence sex differences in the reinforcement enhancing effects of nicotine and how those effects may influence nicotine self-administration.

After conditioning, the value of the cue was tested in all animals. Responding for the cue was equal across groups that had previously responded for the cue alone (in the presence of saline) or responded for the cue in the presence of non-contingent nicotine. This suggests non-contingent nicotine enhances the reinforcing value of the cue only while nicotine is on board, consistent with Chaudhri et al. (2006), and does not result in conditioning of the cue. Additionally, a history of non-contingent nicotine does not appear to have long-lasting effects that influence the reinforcing value of a novel cue. Responding during the testing phase was significantly greater in animals previously responding for contingent nicotine and cue presentations as compared to animals who responded for the cue alone. Through pairing with nicotine delivery, the cue became a conditioned reinforcer and continued to elicit high levels of responding even in the absence of nicotine.

Unexpectedly, responding for the cue was similarly high in animals previously responding for no reinforcement (Activity-Only) or responding for nicotine infusions (NIC-Only) in the absence of the cue. In both the Activity-Only and NIC-Only groups, heightened responding for the cue during the acquisition of a new response phase may be the result of responding for a novel stimulus with reinforcing properties. While Nic-Only, NIC+CS, and Activity-Only animals eventually responded similarly for the CS, it is interesting to assess the trajectory of responding. On the first day of the acquisition of a new response phase, NIC-Only animals responded more than NIC+CS animals ($F=5.9$, 1 d.f., $p=0.018$) and Activity-Only animals

($F=11.3$, 1 d.f., $p=0.002$). Activity-Only animals increased responding across the phase, eventually reaching levels of responding similar to both NIC-Only and NIC+CS animals. For Activity-Only animals, the increase in responding for the CS may simply indicate acquisition of responding for a reinforcer. Previous research has established novel contexts enhance reward or reinforcement (Guitart-Masip et al., 2010; Lisman & Grace, 2005). For NIC-Only animals, the current study indicates this effect may be further enhanced by previous exposure to nicotine. It is important to note a lack of sex differences in these findings.

Determining how nicotine-related cues become conditioned reinforcers is an important part of understanding how smoking-related cues drive smoking behaviors. The current study indicates cues paired with nicotine become conditioned reinforcers over time, and consequently, will support responding for the cue in the absence of nicotine. This finding is consistent with research indicating smokers will continue to smoke denicotinized cigarettes, even when nicotine is being delivered via transdermal nicotine patch (Donny & Jones, 2009).

The a priori hypothesis of the current study predicted greater conditioning effects in females, as compared to males. However, results from the conditioning test did not reveal sex differences. Though other researchers have shown sex differences in responding to non-nicotine cues (Perkins et al., 2001; Perkins et al., 2002; Perkins et al., 2006), the current study indicates these differences are not a result of conditioning. The lack of differences is unlikely related to power to detect differences, as group sizes in this study were large and the findings were consistent across Study 2 and Study 3. The design of the current study allowed for systematic testing of sex differences in acquisition of self-administration during the training phase. As evidenced by a lack of linear day*sex interaction, males and females acquired self-administration at similar rates across the study. This finding may not generalize to the acquisition of responding

for a CS of greater value or a strong US (i.e. a higher dose of nicotine). Indeed, it is possible other researchers have detected sex differences in reinforcement because they use instantaneous measures of reinforcement (i.e. self-report) that are more sensitive to reinforcement enhancement (i.e. pharmacological state) instead of measures of reinforcement assessed in the absence of nicotine (i.e. during the acquisition of a new response).

3.1 LIMITATIONS AND FUTURE DIRECTIONS

The mechanism through which cues become differentially important in female smokers remains unclear. Though the current study fails to find differences in the conditioning of a weakly reinforcing cue with nicotine, it does not address the effects of nicotine conditioning on conditioned reinforcers with stronger pre-conditioning reinforcing properties. Nor does the current study assess the effects of a longer conditioning history. A wider selection of reinforcers should be examined to determine whether the initial strength of the conditioned reinforcer (cue) influences the effects of nicotine conditioning. Variations in the length of conditioning history should also be assessed. Similarly, additional doses of nicotine (strengths of the unconditioned reinforcer) should be examined to determine the relationship between the strength of the unconditioned reinforcer and the level of conditioning. The current study did not find sex differences in conditioning, as indicated in the acquisition of the new response phase. However, comparisons between the NIC+CS and NIC-Only groups suggest males are more sensitive to the conditioning of nicotine-associated cues. Research should continue to find more sensitive measures of assessing conditioning that allow for testing the value of a conditioned reinforcer in the absence of confounding variables such as drug state.

APPENDIX A

TABLES

A.1 TABLE 1. STUDY 1 GROUP ASSIGNMENTS

	<i>CUE</i>	<i>NO STIM</i>
<i>Males</i>	<i>n=15</i>	<i>n=15</i>
<i>Females</i>	<i>n=15</i>	<i>n=15</i>

A.2 TABLE 2. STUDY 2 GROUP ASSIGNMENTS

	<i>NIC+CS</i>	<i>NIC-Only</i>	<i>CS-Only</i>	<i>YN+CS</i>
<i>Males</i>	<i>n=6</i>	<i>n=10</i>	<i>n=11</i>	<i>n=10</i>
<i>Females</i>	<i>n=10</i>	<i>n=11</i>	<i>n=12</i>	<i>n=8</i>

A.3 TABLE 3. STUDY 3 GROUP ASSIGNMENTS

	<i>NIC+CS</i>	<i>NIC-Only</i>	<i>CS-Only</i>	<i>YN+CS</i>	<i>Activity-Only</i>
<i>Males</i>	<i>n=11</i>	<i>n=9</i>	<i>n=12</i>	<i>n=12</i>	<i>n=11</i>
<i>Females</i>	<i>n=11</i>	<i>n=10</i>	<i>n=11</i>	<i>n=8</i>	<i>n=11</i>

A.4 TABLE 4. PAIRWISE COMPARISONS OF REINFORCERS EARNED DURING FR1 RESPONDING

	<u>NIC+CS v.</u> <u>CS</u>	<u>NIC+CS v.</u> <u>NIC</u>	<u>NIC v.</u> <u>Activity</u>	<u>NIC+CS v.</u> <u>ACT</u>	<u>NIC v. CS</u>	<u>NIC+CS v.</u> <u>YN+CS</u>	<u>YN+CS v.</u> <u>CS</u>	<u>CS v.</u> <u>Activity</u>
<u>Day (Within-Subject)</u>	17.3***	46.8***	9.3**	14.9***	2.5	85.2***	17.4***	7.5**
<u>Day*Group</u>	42.5***	5.1*	17.5***	26.7***	16.5***	0.3	47.3***	1.8
<u>Day*Sex</u>	1.0	0.1	0.0	1.7	0.2	2.5	4.2*	1.3
<u>Day*Run</u>	2.7	0.1	-	-	0.6	1.9	1.8	-
<u>Day*group*Sex</u>	0.0	0.2	0.3	0.2	0.5	0.4	0.5	0.0
<u>Day*Group*Run</u>	0.2	4.1*	-	-	4.4*	0.1	0.0	-
<u>Day*Sex*Run</u>	0.2	0.1	-	-	0.0	2.2	2.0	-
<u>Day*Group*Sex*Run</u>	0.1	0.2	-	-	0.0	0.5	1.7	-
<u>Group</u>	52.3***	34.6***	43.6***	134.9***	0.1	1.7	23.2***	85.9***
<u>Sex</u>	0.5	0.4	4.8*	1.4	6.3*	0.1	1.0	8.8**
<u>Run</u>	4.7	0.0	-	-	5.5*	1.7	0.4	-
<u>Group*Sex</u>	3.1	6.4*	0.8	0.3	2.2	1.6	0.0	0.5
<u>Group*Run</u>	6.3*	12.6***	-	-	4.0*	4.6*	0.1	-
<u>Sex*Run</u>	6.3*	1.8	-	-	1.2	0.8	0.3	-
<u>Group*Sex*Run</u>	0.4	1.2	-	-	0.7	2.0	1.3	-

All reported values are F statistics.

*=p<0.05

**=p<0.01

***=p<0.001

A.5 TABLE 5. PAIRWISE COMPARISONS OF REINFORCERS EARNED DURING FR2 RESPONDING

	<u>NIC+CS v.</u> <u>CS</u>	<u>NIC+CS v.</u> <u>NIC</u>	<u>NIC v.</u> <u>ACT</u>	<u>NIC+CS v.</u> <u>ACT</u>	<u>NIC v. CS</u>	<u>NIC+CS v.</u> <u>YN</u>	<u>YN+CS v.</u> <u>CS</u>	<u>CS v. ACT</u>
<u>Day (Within-Subject)</u>	43.1***	36.3***	9.7**	11.2**	16.2***	23.9***	5.3*	1.3
<u>Day*Group</u>	18.2***	2.9	10.2**	11.8***	3.3	10.9**	0.0	1.5
<u>Day*Sex</u>	3.3	0.1	0.2	0.9	0.7	0.4	0.1	0.8
<u>Day*Run</u>	5.361*	2.2	-	-	0.1	2.9	0.2	-
<u>Day*group*Sex</u>	1.5	3.7	1.1	0.2	2.0	2.8	1.0	0.0
<u>Day*Group*Run</u>	3.1	2.0	-	-	0.0	1.9	0.0	-
<u>Day*Sex*Run</u>	0.6	0.1	-	-	0.3	1.2	0.2	-
<u>Day*Group*Sex*Run</u>	0.9	0.9	-	-	0.1	0.0	0.5	-
<u>Group</u>	100.2***	35.5***	46.8***	162.3***	5.5*	10.8**	60.0***	58.0***
<u>Sex</u>	0.6	0.3	0.8	0.9	4.5*	0.1	4.2*	6.8*
<u>Run</u>	6.6*	1.6	-	-	0.5	2.2	0.4	-
<u>Group*Sex</u>	1.5	4.0*	0.2	0.2	2.6	3.2	2.0	3.1
<u>Group*Run</u>	5.3*	5.7*	-	-	1.0	6.2*	0.9	-
<u>Sex*Run</u>	6.7*	0.2	-	-	0.0	0.8	0.3	-
<u>Group*Sex*Run</u>	0.4	3.9	-	-	4.7*	3.1	3.7	-

All reported values are F statistics.

*=p<0.05

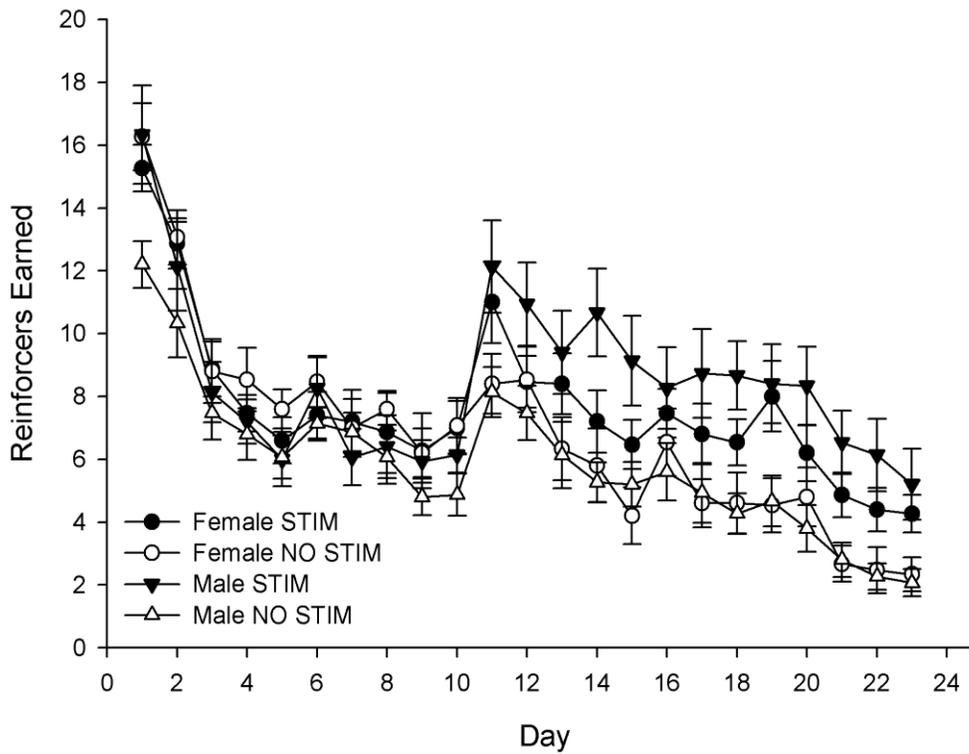
**=p<0.01

***=p<0.001

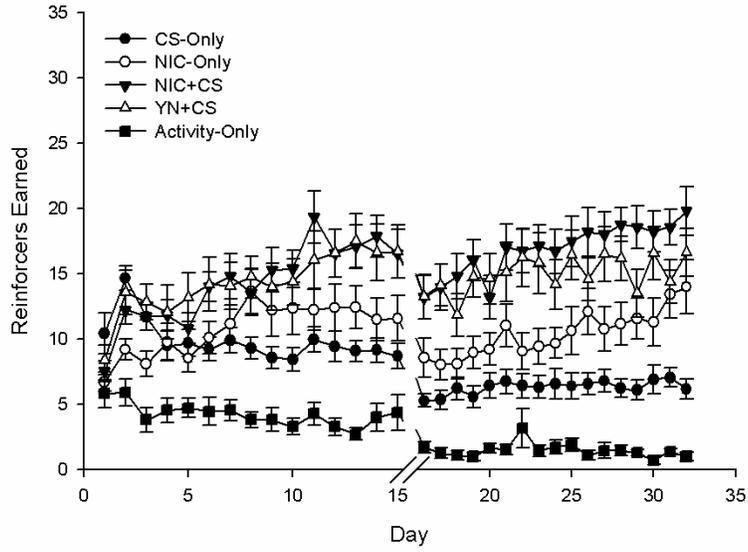
APPENDIX B

FIGURES

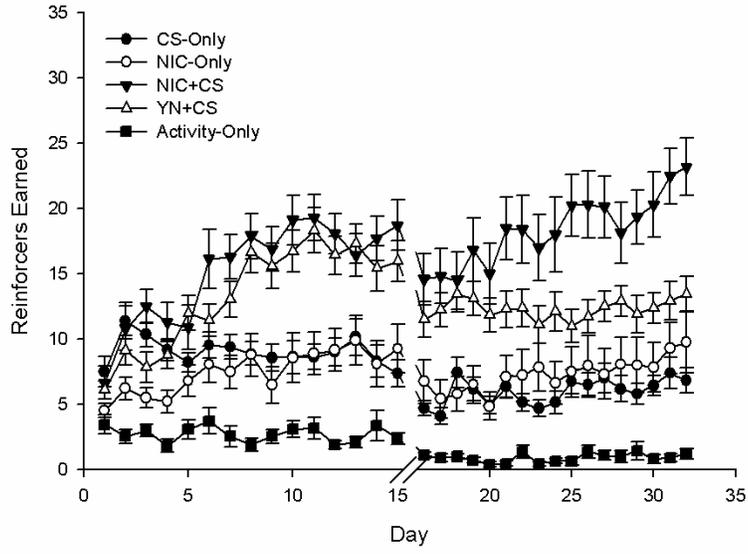
B.1 FIGURE 1



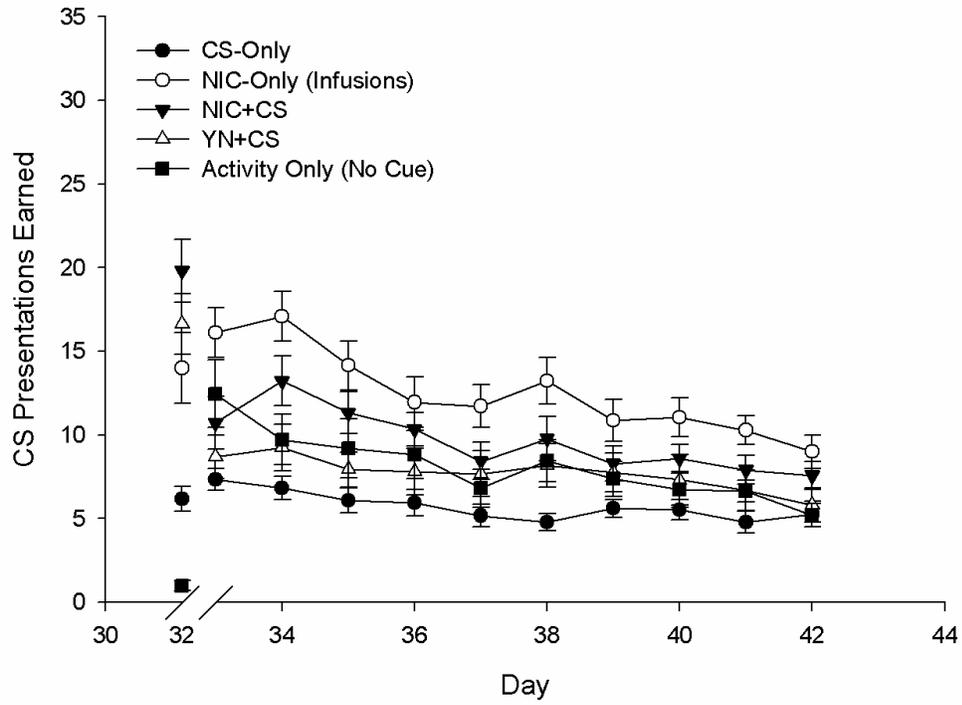
B.2 FIGURE 2



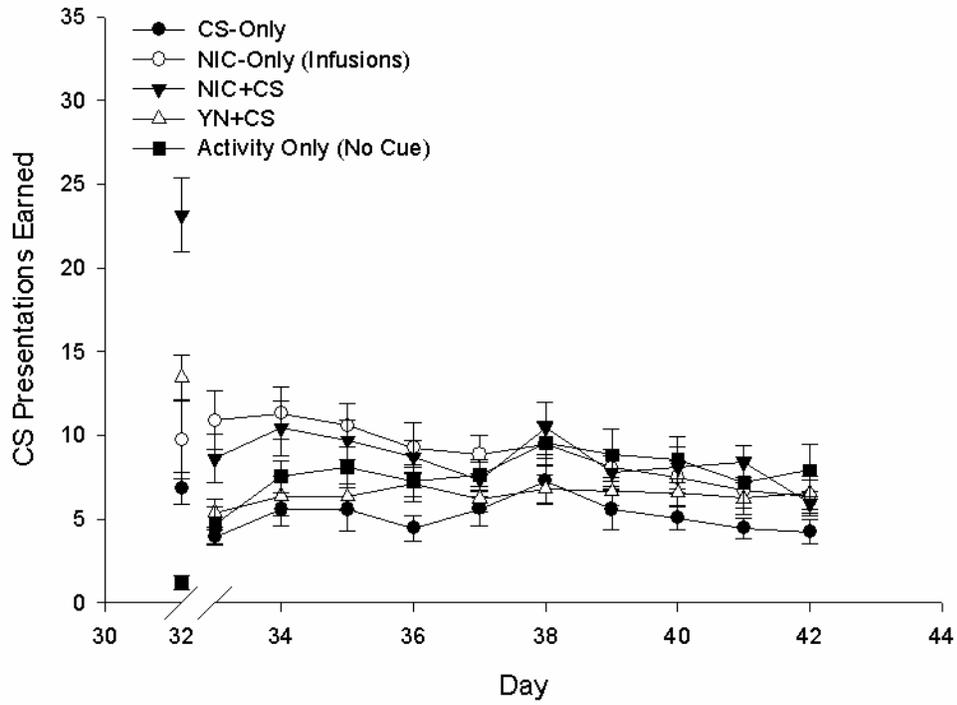
B.3 FIGURE 3



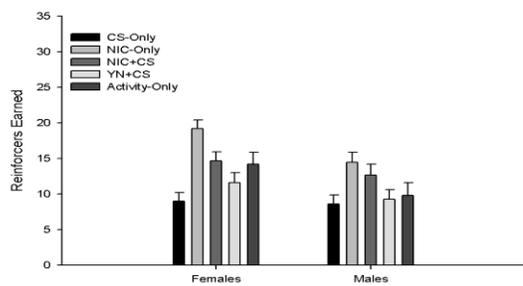
B.4 FIGURE 4



B.5 FIGURE 5



B.6 FIGURE 6



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