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Behavioral effects of nicotine withdrawal in adult male and female rats

Kristen R. Hamilton *, Sarah Shafer Berger, Michael E. Perry, Neil E. Grunberg

Uniformed Services University (USU), Department of Medical and Clinical Psychology, 4301 Jones Bridge Road, Bethesda, Maryland 20814, United States

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ABSTRACT

Nicotine withdrawal may differ between men and women but clinical reports are inconsistent. Two experiments were conducted to examine behavioral effects of nicotine withdrawal in male and female adult rats in dimly-lit and brightly-lit environments. Ninety-six Sprague–Dawley male and female rats received 7 days continuous subcutaneous infusion via ALZET osmotic minipumps filled with saline or 3.16 mg/kg/day nicotine hydrogen tartrate expressed as base. Behavioral observations were made before, during, and after drug administration. During observations, occurrences of empty-mouth-chewing, whole-body-shakes, abnormal grooming, abnormal posture/movement, diarrhea, ptosis, eyeblinks, and any other abnormal behaviors were counted. Cessation of nicotine administration upon pump removal caused a significant increase in withdrawal behaviors in males and females in both environments. In the dimly-lit environment, females showed more withdrawal behavior than males; there was no sex difference in the brightly-lit environment. Males that had received nicotine displayed more withdrawal behavior in the brightly-lit environment than in the dimly-lit environment, while females that had received nicotine displayed similar amounts of withdrawal behavior in both environments. Behavioral symptoms of withdrawal may be more affected by the environment in male rats than in female rats. These experiments are the first to compare nicotine withdrawal in adult male and female rats.

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1. Introduction

Cigarette smoking is the leading cause of preventable death in the United States, and leads to significant health consequences, including cardiovascular diseases, cancers, and respiratory diseases (Centers for Disease Control [CDC], 2007). Despite these health consequences, one out of every five adults in the U.S. smokes cigarettes (CDC, 2007). People continue to smoke cigarettes largely because of nicotine, a highly addictive drug that plays a major role in reinforcing the maintenance of tobacco use (Grenhoff and Svensson, 1989; Grunberg et al., 2000; Henningfield and Benowitz, 1995; Koob and LeMoal, 2008; United States Department of Health and Human Services [USDHHS], 1988).

Cessation of nicotine administration results in nicotine withdrawal symptoms and behaviors in humans and animals. Marked nicotine withdrawal in humans lasts for approximately 10 days, and symptoms include tension, irritability, headaches, and increased appetite. Body weight gain and craving for cigarettes last for roughly a year (e.g., Hughes et al., 1990; Koob and LeMoal, 2008; Shiffman et al., 2006; USDHHS, 1988). The occurrence of withdrawal symptoms upon cessation of drug administration provides a useful measure of addiction. Malin et al. (1992) developed an animal model to examine nicotine withdrawal in rats. Previous work from the Malin group was focused on morphine dependence, in which it was discovered that some of the most widespread and useful models of morphine dependence were those in which rats spontaneously exhibited quantifiable unusual behaviors during abstinence (Gianutsos et al., 1975; Malin et al., 1988). With the aim of developing a laboratory model of nicotine withdrawal, the Malin group conducted extensive pilot studies in which they took various physiological measurements and recorded all countable behavioral events before, during, and after nicotine infusion (Malin, 2001). They identified certain behaviors, termed "somatic behavioral signs," as being selectively elevated during the withdrawal phase, particularly whole body shakes, abnormal grooming, abnormal posture or movement, ptosis (slackening of the jaw), empty-mouth chewing/teeth chattering, eyeblinks, and diarrhea.

Several lines of evidence support the validity of the present model as a representation of nicotine withdrawal syndrome (Kenny and Markou, 2001). First, when nicotine is chronically administered and then withdrawn from rats, they display more somatic behavioral signs than when these same subjects were nicotine naive, immediately prior to the termination of nicotine administration, after the recovery from withdrawal, or compared to saline-treated control rats (Malin et al., 1992). Second, the severity of the somatic behavioral signs was proportional to the amount of nicotine to which the animal was exposed, with animals receiving higher concentrations of nicotine displaying more behavioral signs. Third, nicotine administration reverses withdrawal behavioral signs in rats undergoing nicotine

* Corresponding author. Tel.: +1 301 295 9670; fax: +1 301 295 3304.
E-mail address: khamilton@usuhs.mil (K.R. Hamilton).

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withdrawal, which demonstrates that tonic activation of nicotinic cholinergic receptors (nAChR), which are upregulated when addiction develops, is critical to prevent the somatic behavioral symptoms (Malin et al., 1992). In addition, administration of bupropion, a compound that is clinically efficacious in the treatment of nicotine dependence, reverses both somatic and affective signs of nicotine withdrawal (Cryan et al., 2003).

Malin's rodent model of nicotine withdrawal has proven to be reliable with rats and mice because it has produced consistent results across a number of experiments of nicotine withdrawal from the Malin group (1993, 1994, 1996, 1998; Malin, 2001) and other laboratories (Carboni et al., 2000; Epping-Jordan et al., 1998; Hildebrand et al., 1997, 1998; Kota et al., 2007, 2008; O'Dell et al., 2004; Phillips et al., 2004; Watkins et al., 2000). Additional somatic signs of nicotine withdrawal reported include escape attempts, foot licks, genital licks, and writhes (e.g., O'Dell et al., 2004; Skjei and Markou, 2003). Some work with rodent models has been focused on individual differences, specifically age differences, and their effect on nicotine withdrawal (Kota et al., 2007, 2008; O'Dell et al., 2004, 2006). However, no published studies have used this model to examine nicotine withdrawal in female rats.

In the U.S. men are more likely to smoke cigarettes (23.9%) than women (18.1%) (CDC, 2007), and men smoke more cigarettes than women (Grunberg et al., 1991; Perkins, 1996). Yet, women report less success quitting smoking than do men (Perkins, 2001; Swan et al., 1997; Wetter et al., 1999). Some investigators report more self-reported nicotine withdrawal symptoms in women than men (e.g. Shiffman, 1979) but others report no gender differences in withdrawal (e.g., Hughes and Hatsukami, 1992; Svikis et al., 1986). No reports indicate greater withdrawal symptoms in men than women. It has been noted that the studies in which women self-report greater withdrawal severity than men were retrospective (Hughes et al., 1990). Pomerleau et al. (1994) conducted a retrospective and prospective study of self-reported nicotine withdrawal symptoms in women and men. Women reported more withdrawal than men retrospectively, but there were no gender differences in reported withdrawal symptom severity in the prospective portion of the study (i.e., while in withdrawal). Based on the available literature, it is unclear whether there are sex differences in nicotine withdrawal in humans.

Research on nicotine's effects in rodent models reports sex differences depending on the measures studied. For example, female rats are more sensitive than male rats to effects of nicotine on body weight, feeding, pre-pulse inhibition of the acoustic startle reflex, antinociception, and behavioral sensitization (Chiari et al., 1999; Craft and Milholland, 1998; Faraday et al., 1999; Grunberg et al., 1986; Harrod et al., 2004). However, female rats are less sensitive to the discriminative effects of nicotine (Schechter and Rosencrans, 1971). Studies in mice suggest that females are less sensitive to nicotine-induced suppression of Y-maze activity, nicotine-induced increase in active avoidance learning, withdrawal from nicotine, and nicotine's positive and rewarding effects (Hatchell and Collins, 1980; Kota et al., 2007, 2008; Yilmaz et al., 1997). However, nicotine withdrawal effects have not been directly compared in male and female mice, and sex differences in nicotine withdrawal have not been investigated in a rat model.

Environment may also be an important variable to examine with regard to nicotine withdrawal. Smoking-related stimuli, smoking-related activities, and environmental context may be more important, especially for women (Parrott and Craig, 1995; Perkins, 1996). In addition, the extent to which a given environment is stressful or not may be relevant to nicotine withdrawal because stress is associated with increased smoking (e.g., George et al., 2007; Grunberg and Baum, 1985; Jarvik et al., 1977; Kassel et al., 2003; Pomerleau and Pomerleau, 1987; Schachter et al., 1977).

Details about the environmental conditions in which withdrawal behavioral observations were conducted were not reported in previous withdrawal research (e.g., Kota et al., 2007, 2008; Malin et al., 1993, 1994, 1996, 1998; Malin, 2001; O'Dell et al., 2004). However, it was revealed in personal communications with Malin and O'Dell that the behavioral observations took place in a brightly-lit room in cages without bedding. In the present research, nicotine withdrawal in adult male and female rats was examined in two different environments. The animal model allowed for random assignment of subjects to drug groups, manipulation of environment, and assessment of nicotine withdrawal based on observed behaviors.

2. Experiment 1: adult females and males observed in a dimly-lit environment in cages with bedding

2.1. Overview

The purpose of this experiment was to examine the effect of nicotine withdrawal in male and female adult rats in a dimly-lit environment in cages with bedding. Withdrawal behaviors identified by Malin et al. (1992) were observed and recorded before, during, and after administration of nicotine via osmotic minipumps. In the present experiment, the observation room was dark to be similar to the home-cage environment. The observation cages contained bedding material and were identical to home cages, and the observations were made during the rats' dark, active phase. There were four behavioral observation sessions during the course of the experiment. Observations were conducted once weekly during the baseline and nicotine phases, and twice during the cessation phase: the first withdrawal behavioral observation occurred at 20 h post pump removal, and the second observation occurred at 44 h post pump removal. The present experiment was a 2 (male, female)×2 (nicotine, saline) mixed model with repeated measures. The dependent variables were observed withdrawal behaviors and open field locomotor activity.

2.2. Subjects

Subjects were 24 female and 24 male Sprague–Dawley rats obtained from Charles River Laboratories (Wilmington, MA). Animals were individually housed in standard polycarbonate shoebox cages (42×20.5×20 cm) on hardwood chip bedding (Pine-Dri). Animals had continuous access to rodent chow (Harlan Teklad 18% Protein Rodent Diet 2018) and water during all phases of the study in the home cages. Housing rooms were maintained at 23 °C at 50% humidity on a 12 hour light/dark cycle (lights off at 0800 h). Rats were approximately 70 days old at the start of the experiment—an age in rats that is analogous to young adulthood (Douglas et al., 2004). At the beginning of the experiment, the females weighed an average of 186.5 g and the males weighed an average of 303.7 g. This experimental protocol was approved by the USU Institutional Care and Use Committee and was conducted in full compliance with the NIH Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985).

2.3. Methods

2.3.1. Baseline phase

The baseline phase lasted for one week (7 days) after the rats' arrival. In the first two days after arrival, rats were gentled by being held and petted for 2 min each so that they would become accustomed to handling by humans, and were acclimated to the activity chambers. Daily collection of estrous samples began on the third day of the baseline phase for females. On the sixth day, locomotor activity was measured by placing the rats into individual electronic physical activity monitoring chambers of the Omnitech/Accuscan Electronics Digiscan infrared photocell system (Test box model RXYZCM [16 TAO]; Omnitech/Accuscan Electronics, Columbus, OH) for 1 h to measure open field locomotor activity. Baseline behavioral observations were conducted on the seventh day of the baseline phase.

During the observation period, raters recorded occurrences of withdrawal behaviors (Malin et al., 1992, 1994, 2006; Phillips et al., 2004; O'Dell et al., 2004, 2006). All behavioral observations took place during the dark (active) phase of the rats' light cycle. Observations were conducted in a dark room illuminated by one 60 watt light bulb in a corner, and each observation period lasted for 15 min. The observation room was illuminated at 4.30 lx (Advanced Light Meter, Model No. 840022, Sper Scientific Ltd.). Observations were conducted in a dimly-lit room in cages with bedding to model the home environment as closely as possible, and to be consistent with other experiments conducted in our laboratory (Phillips et al., 2004). Occurrences of six specific types of behavior were quantified by observers: abnormal posture or movement, abnormal grooming, whole body shakes, ptosis, empty-mouth chewing/teeth chattering, and diarrhea. Abnormal postures or movements could include writhing or twisting of the body while in a sitting or standing position. Abnormal grooming is especially persistent or rough grooming behavior which may include chewing of the forepaws or other body parts, and vigorous washing of the face and body. Every 10 second episode of abnormal grooming was considered a discrete occurrence of the behavior. Ptosis is a slackening or relaxing of the facial muscles. Empty-mouth chewing or teeth chattering is rapid chattering of the teeth or empty-mouth chewing. Unusual behaviors that were not included in one of the six predefined categories were counted as episodes of "other" behaviors (e.g., foot licks, genital licks, gasps, yawns). Each rat was observed by two trained raters. Inter-rater reliability was approximately 90%.

Estrous measurements were also conducted on the female rats. Estrous measurements were made daily at 0900 h for 21 days, beginning three days after the rats' arrival. A flushing technique was used to collect vaginal epithelial cells from each rat (Wayneforth and Flecknell, 1992). The cells were placed on microscope slides that were later dipped into methanol (7 min), water three times (3 min each), hematoxylin (4 to 5 min), alcohol (1 to 2 min), eosin (2 to 5 min), water (briefly), alcohol three times (1 to 2 min each), and xylene (1 min). Cover slips were fixed to the slides using Permount, and the slides were allowed to dry. After drying, slides were viewed by independent raters under a Reichert-Jung microscope (Series 150) at 40× magnification, and stage of estrous cycle was recorded.

2.3.2. Drug administration phase

The drug administration phase began after the pump implant. During this one-week phase, rats received continuous administration of 3.16 mg/kg/day nicotine bitartrate expressed as base or physiological saline (0.9% NaCl) at 5.25 µl/h. Daily estrous measurements were continued at 0900 h. Behavioral observations took place on the sixth day of the drug administration phase as described above.

Subjects were anesthetized individually in a plastic chamber with a continuous flow of oxygen (flow rate: 0.5 to 1.0 l/min) and 2 to 4% isoflurane gas into the chamber to induce anesthesia. Pump implant occurred between 1200 and 1400 h on the eighth day after the rats' arrival. The rats' anesthesia-induced unconscious state was maintained during the implant via a nose cone and tube that delivered a combination of 0.25 to 3% isoflurane and oxygen from the induction chamber. ALZET osmotic minipumps (Model 2ML2, DURECT Corporation, Cupertino, CA) filled with nicotine hydrogen tartrate (bitartrate) solution or 0.9% NaCl (physiological saline) were implanted SC between the withers, based on procedures of Grunberg (1982). The pumps delivered a continuous flow of 3.16 mg/kg/day nicotine bitartrate (expressed as a base) at approximately 5 µl/h. This dosage has been used in previous studies of nicotine withdrawal (e.g., Malin et al., 1992, 1994, 2006; O'Dell et al., 2004, 2006; Phillips et al., 2004). Observations during the drug administration phase were conducted in an identical manner to observations during the baseline phase. The drug administration phase ended with the pump explant, which took place from 1600 h to 2000 h on the last day of the nicotine phase, one week after pump implant.

2.3.3. Withdrawal phase

After seven days of nicotine or saline administration, animals were anesthetized (as above) and pumps were explanted. Withdrawal phase began immediately after pump explant. Rats were observed 20 h after pump removal, in the middle of the optimal 18 to 22 h window for observing withdrawal behaviors described by Malin et al. (1992, 1994, 2006), O'Dell et al. (2004, 2006), and Phillips et al. (2004). The isoflurane anesthesia did not affect withdrawal behavior on the observation days because the half-life ($t_{1/2}$) of isoflurane in a rat that is 5–6 months old is about 7–9 min, and the slow wash-out from the brain, which is thought to involve elimination from intracranial fatty tissue, takes 100–115 min (Chen et al., 1992). Conducting the first observation at 20 h post-pump removal allowed sufficient time for the isoflurane to be eliminated from the body. The locomotor activity parameters of horizontal and vertical activity and center time were collected for 1 h in the locomotor chambers on Withdrawal Day One immediately following observations. There was also a second withdrawal observation that took place 24 h after the first withdrawal observation. Estrous measurements continued for nine days after the first withdrawal day.

Observations during the withdrawal phase were conducted in an identical manner to observations during the baseline and nicotine phases. Measurements of locomotor activity were collected during the withdrawal phase in an identical manner to locomotor activity measurement collection during the baseline phase.

2.4. Experiment 1: results

2.4.1. Data analytic strategy

Withdrawal behavior data were analyzed with Analyses of Variance (ANOVAs) at baseline and during saline or nicotine administration to determine whether differences in withdrawal behaviors existed prior to nicotine withdrawal. Withdrawal behavior data on cessation days were analyzed with repeated-measures Analyses of Covariance (ANCOVAs) using mean baseline behavior as a covariate. Additionally, ANCOVAs using baseline withdrawal behaviors as the covariate were conducted for each of the two withdrawal days. Estrous data were analyzed using a mixed model approach (Arnold, 1992). The phases of the estrous cycle were dummy coded and the data were analyzed to determine whether amount of withdrawal behavior displayed was related to estrous cycle phase. The dependent variables within open field were center time, vertical activity, and horizontal activity, and they were analyzed using a repeated-measures ANOVA with drug condition as the between-subjects factor. All analyses were two-tailed with $p<0.05$.

2.4.2. Behavioral signs of withdrawal

At baseline there were no differences between drug conditions, but there was a sex difference in total behaviors that were later used to index nicotine withdrawal [$F(1, 44)=7.44$, $p<0.01$], with males having more behaviors than females. There were no main effects of drug condition or sex during the drug administration phase.

Repeated-measures ANCOVA revealed a main effect of drug condition during the withdrawal phase [$F(1, 44)=16.39$, $p<0.01$], with rats that had received nicotine displaying more behaviors than rats that had received saline. There was also a main effect of sex during the withdrawal phase [$F(1, 44)=29.73$, $p<0.01$], with females displaying more withdrawal behaviors than males. There was no sex × drug condition interaction in Experiment 1. The results of Experiment 1 are displayed in Fig. 1.

For Withdrawal Day One, an ANCOVA using baseline withdrawal behaviors as a covariate revealed a significant main effect for drug condition [$F(1, 44)=15.31$, $p<0.01$], with rats that had received nicotine displaying significantly more withdrawal behaviors than rats that had received saline, and a main effect for sex [$F(1, 44)=29.33$,

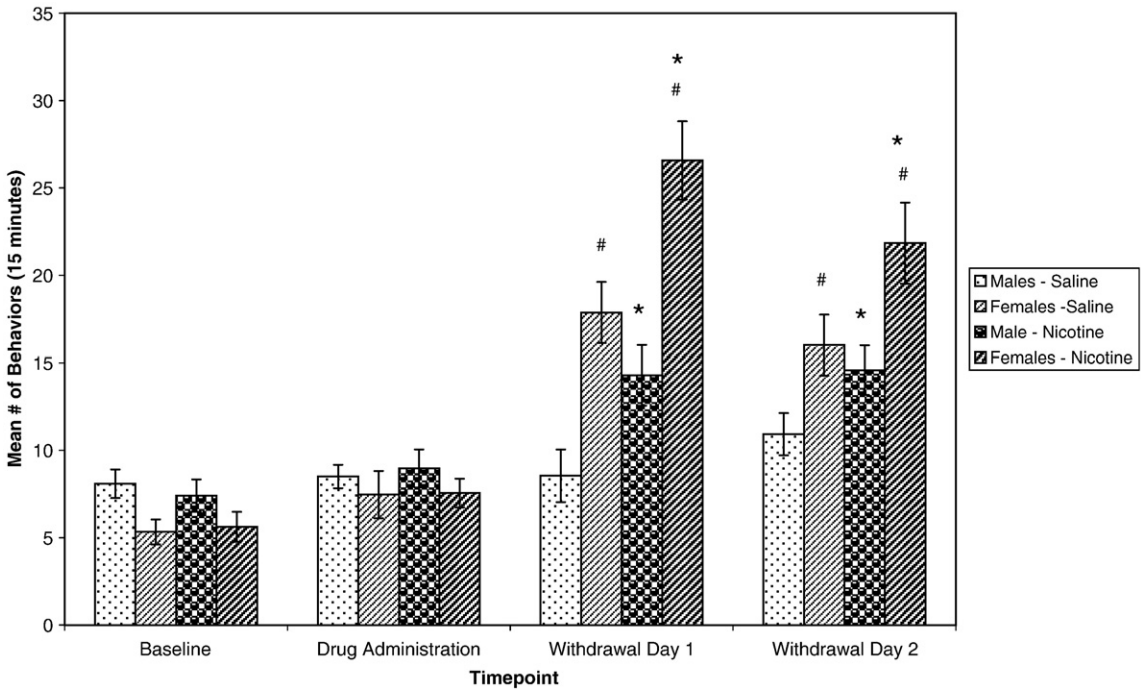

Fig. 1. Mean number of total withdrawal symptom behaviors (mean±S.E.M.) observed in all rats across four 15 minute observation periods. Rats were observed in 42 cm×20.5 cm×20 cm cages with bedding in a dimly-lit room. The * symbol denotes significance when compared to the same-sex control condition, and the # symbol denotes significance when compared to the opposite sex within the same drug condition.

$p<0.01$], with females in both drug conditions displaying significantly more withdrawal behaviors than males in the corresponding conditions. There was no sex×drug condition interaction.

For Withdrawal Day Two, an ANCOVA using baseline withdrawal behaviors as a covariate revealed a significant main effect for drug condition [$F(1, 44)=7.66$, $p<0.01$], with rats that had received nicotine displaying significantly more withdrawal behaviors than rats that had received saline, and a main effect for sex [$F(1, 44)=12.78$, $p<0.01$] with females in both conditions displaying significantly more withdrawal behaviors than males in the corresponding conditions. There was no sex×drug condition interaction.

2.4.2.1. Individual withdrawal behaviors. There was a main effect for sex on the occurrence of whole body shakes [$F(1, 44)=43.870$, $p<0.01$], with females in both drug conditions displaying more whole body shakes than males in the corresponding drug conditions.

There were main effects of drug condition [F (1, 44)=8.576, $p<0.01$] and sex [F (1, 44)=8.002, $p<0.01$] on the occurrence of episodes of abnormal grooming behavior. Rats that had received nicotine displayed more abnormal grooming behavior than rats that had received saline, with females displaying more abnormal grooming behavior than males in the corresponding drug conditions. However, there was no sex×drug condition interaction.

There was a significant main effect of drug condition on abnormal posture [F (1, 44)=10.801, $p<0.01$], with rats that had received nicotine displaying more abnormal postures than rats that had received saline.

There were main effects of drug condition [F (1, 44)=4.834, $p<0.05$] and sex [$F(1, 44)=32.834$, $p<0.01$] on behaviors categorized as "other," as well as a drug×sex interaction [$F(1, 44)=5.187$, $p<0.025$]. Rats that had received nicotine displayed more "other" behaviors than those that had received saline, and females displayed more "other" behaviors than males, with females that had received nicotine displaying more "other" behavior than any other group.

There were main effects of drug condition [F (1, 44)=22.749, $p<0.01$] and sex [F (1, 44)=7.969, $p<0.01$] on eyeblinks, with males displaying more eyeblinks than females, and rats that had received nicotine displaying more eyeblinks than rats that had received saline. In addition, there was a drug×sex ordinal interaction, in which males that had received nicotine displayed more eyeblinks than all other groups.

2.4.3. Estrous

Estrous cycle was not significantly associated with withdrawal behavior.

2.4.4. Locomotor activity

There was no main effect for drug or sex on locomotor activity during any phase of the experiment. Female and male saline rats did not differ from the female and male nicotine rats on measures of horizontal activity, vertical activity, or center time.

2.5. Experiment 1: summary of results

Experiment 1 was conducted in an environment that was similar to home cages. Observations were conducted in a darkened room in cages with bedding, and the observation period was 15 min long. Rats that had received nicotine displayed more withdrawal behaviors than rats that had received saline. Additionally, females displayed more overall withdrawal behaviors than males on Withdrawal Days 1 and 2, regardless of drug condition, but there was no sex×drug interaction.

There were no differences in locomotor activity between the saline and nicotine groups in Experiment 1. Therefore, it is unlikely that differences in withdrawal behaviors observed between the saline and nicotine conditions were the result of changes in overall locomotor activity, but instead resulted from increases in the specific nicotine withdrawal behaviors. Estrous cycle phase was not significantly associated with withdrawal behavior in Experiment 1.

3. Experiment 2: adult females and males observed in a brightly-lit environment in cages without bedding

3.1. Overview

The purpose of this experiment was to examine male and female rats' nicotine withdrawal in an environment that differed from that of Experiment 1. Because rats are nocturnal animals that naturally avoid the light, exposure to bright light may be a stressor for rats and has been used as a stressor in experimental investigations (e.g., Frye and Orecki, 2002; Slawecki, 2005). In Experiment 2, nicotine withdrawal was observed in a brightly-lit environment illuminated at 311.5 lx (Advanced Light Meter, Model No. 840022, Sper Scientific Ltd.) by overhead fluorescent lights for 20 min in slightly larger cages (46 cm×36 cm×20 cm) without bedding, to examine the impact of potentially stressful environmental conditions on nicotine withdrawal and to be consistent with similar experiments conducted by other laboratories (e.g., Malin et al., 1992, 1994, 2006; O'Dell et al., 2004, 2006). In Experiment 1, withdrawal observations were conducted in a dimly-lit room for 15 min in cages with bedding to model the home environment as closely as possible and to be consistent with other experiments conducted in our laboratory (Phillips et al., 2004). Examining nicotine withdrawal in two different environments allowed for comparison with reported experiments in the research literature and addressed the question of whether environmental differences altered effects of nicotine withdrawal in this paradigm. However, withdrawal behavior differences between Experiments 1 and 2 also might reflect the different observation period durations of the two experiments. There were five behavioral observation sessions during the course of the experiment. Observations were conducted once weekly during the baseline and nicotine phases, and three times during the cessation phase: at 20 h, 44 h, and 68 h post pump removal.

3.2. Subjects

Subjects were 24 Sprague–Dawley males and 24 Sprague–Dawley females. All rats were approximately 70 days old at the start of the experiment. Upon arrival, the males' mean weight was 286.77 g, and the females' mean weight was 200.21 g. Housing conditions in Experiment 2 were identical to the housing conditions in Experiment 1. This experimental protocol was approved by the USUHS Institutional Care and Use Committee and was conducted in full compliance with the NIH Guide for Care and Use of Laboratory Animals.

3.3. Experiment 2: methods

3.3.1. Baseline phase

The baseline phase in Experiment 2 was identical to the baseline phase in Experiment 1 with the only exceptions being those listed above (i.e., longer period of observation, a larger observation cage without bedding, observation with overhead lights on). As in Experiment 1, all behavioral observations were conducted during the dark (active) phase of the rats' light cycle.

3.3.2. Drug administration phase

Drug administration, surgical procedures, observations, and locomotor activity measurements were all identical to Experiment 1, except for the differences in observation conditions described above.

3.3.3. Withdrawal phase

Minipumps were explanted from the rats in a surgical procedure identical to that used in Experiment 1. A third withdrawal observation was conducted in Experiment 2, so that rats were observed at 20 h post pump removal, and 24 and 48 h after the first observation. The locomotor activity parameters of horizontal and vertical activity and center time were collected for 1 h in the locomotor chambers on Withdrawal Day One, following observations. Estrous measurements continued for nine days after the first withdrawal day.

Observations during the withdrawal phase were conducted in an identical manner to observations during the baseline phase. As described above, the environment in which withdrawal observations were conducted differed from the observation environment in Experiment 1.

3.3.4. Data analytic strategy

The data analytic strategy for Experiment 2 was identical to that of Experiment 1, except that Withdrawal Day Three was included as a time-point in the repeated-measures ANCOVA. A separate ANCOVA using baseline as a covariate was also conducted for Withdrawal Day Three, as was done for Withdrawal Days One and Two.

3.4. Experiment 2: results

3.4.1. Behavioral signs of withdrawal

There were no differences in behaviors that were later used to index nicotine withdrawal among the groups during the baseline and drug administration phases. There was a main effect of drug condition during the withdrawal phase [$F(1, 44)=18.73$, $p<0.01$], with animals that had received nicotine displaying significantly more withdrawal behaviors than animals that had received saline, but there was no significant effect of sex. The withdrawal behavior results of Experiment 2 are displayed in Fig. 2.

ANCOVA using baseline as the covariate revealed a main effect of drug condition on Withdrawal Day One [$F(1, 44)=29.63$, $p<0.01$], with rats that had received nicotine displaying more withdrawal behaviors than rats that had received saline, but no effect of sex, with males and females displaying similar amounts of withdrawal behaviors.

On Withdrawal Day Two, there was a main effect of drug condition [$F(1, 44)=7.51$, $p<0.01$], with rats that had received nicotine displaying more withdrawal behaviors than rats that had received saline, but no effect of sex. On Withdrawal Day Three, there was a main effect of sex [$F(1, 44)=8.07$, $p<0.01$], with male rats from both drug conditions displaying more withdrawal behaviors than female rats from the corresponding drug conditions, but no effect of drug condition.

3.4.1.1. Individual withdrawal behaviors. When individual behaviors were analyzed separately, there were main effects of drug condition [$F(1, 44)=5.274$, $p<0.03$] and sex [$F(1, 44)=8.148$, $p<0.01$] on abnormal posture/movement, with rats that had received nicotine displaying more episodes of abnormal posture or movement than rats that had received saline, and males displaying more abnormal postures and movements than females. However, there was no sex by drug condition interaction.

There were main effects of drug condition and sex on behaviors categorized as “other,” with rats that had received nicotine displaying more “other” behaviors than rats that had received saline, and males displaying more “other” behaviors than females. There was a drug×sex ordinal interaction, in which males displayed more “other” behaviors than females, but males that had received nicotine displayed more behaviors categorized as “other” than all other groups.

3.4.2. Estrous

Estrous cycle phase was not significantly associated with withdrawal behavior throughout the experiment.

3.4.3. Locomotor activity

There were no differences in locomotor activity between rats that had received nicotine and rats that had received saline.

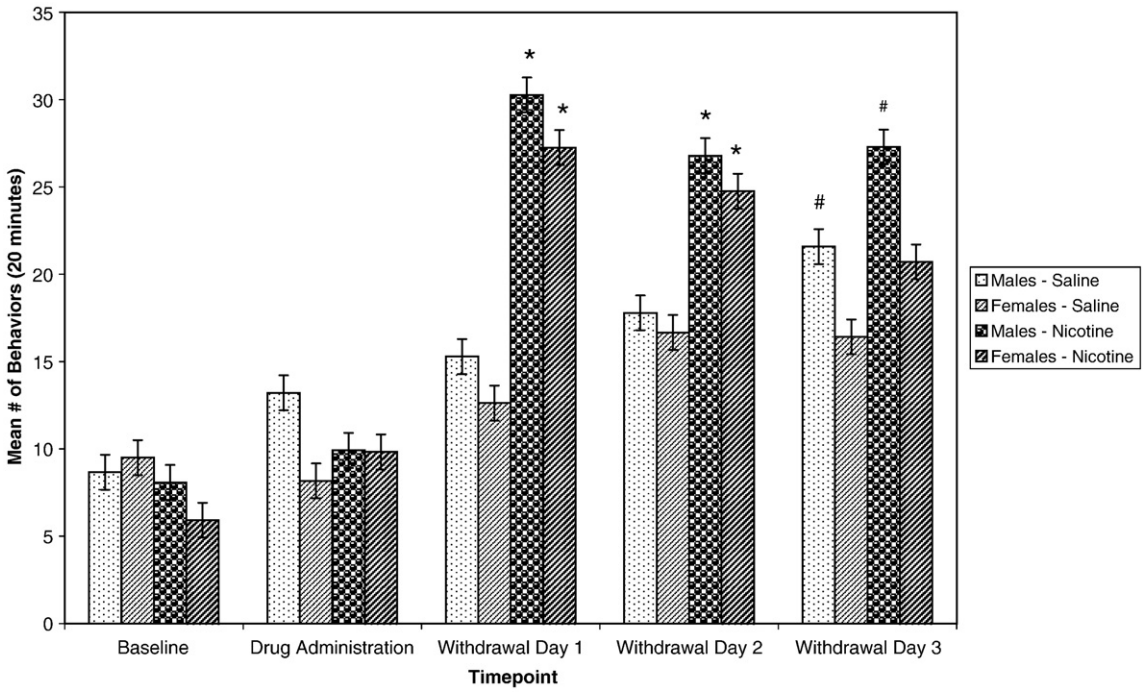

Fig. 2. Mean number of total withdrawal symptom behaviors (mean±S.E.M.) observed in all rats across five 20 twenty minute observation periods. Rats were observed in 46 cm×36 cm×20 cm cages without bedding in a brightly-lit room. The * symbol denotes significance when compared to the same-sex control condition, and the # symbol denotes significance when compared to the opposite sex within the same drug condition.

3.5. Experiment 2: summary of results

In the brightly-lit environment of Experiment 2, cessation of nicotine resulted in marked withdrawal behaviors in male and female rats. The magnitude of males' withdrawal behavior was greater than in Experiment 1, whereas the magnitude of females' withdrawal behavior was similar to that displayed in the dimly-lit observation environment of Experiment 1. There was a main effect of drug condition in both experiments, but the main effect of sex that occurred in Experiment 1 did not occur in Experiment 2.

There were no differences in locomotor activity between the saline and nicotine groups in Experiment 2. Therefore, differences in withdrawal behavior could not be attributed to changes in overall locomotor activity.

Estrous cycle phase was not significantly associated with withdrawal behavior in Experiment 2. The addition of cycle to the mixed model yielded only a 2.8% reduction in total variance.

4. General discussion

In Experiment 1 (dimly-lit environment similar to home cages) female rats displayed more withdrawal behavior after cessation of nicotine than did males. In Experiment 2 (brightly-lit environment dissimilar from home cages) male and female rats displayed similar amounts of withdrawal behaviors. The observation period in Experiment 2 was 5 min longer than the observation period in Experiment 1, which likely contributed to the total amounts of withdrawal behavior observed. To control for the longer observation period used in Experiment 2, ratios were calculated for each observation period in which withdrawal behaviors displayed by the nicotine group were divided by withdrawal behaviors displayed by the saline group during that period. The ratios of withdrawal behaviors for males and females are listed in Table 1. Interestingly, female control rats displayed more behavioral signs of withdrawal in the dimly lit-environment than in the brightly-lit environment, but the female rats that had received nicotine displayed similar amounts of withdrawal behavior in both environments. By contrast, male rats from both drug conditions displayed more behavioral signs of withdrawal in the brightly-lit environment than in the dimly-lit environment, suggesting that the environment had a greater modulating effect on withdrawal behavior in male rats that had received nicotine. This difference is reflected statistically by the fact that the main effect of sex that occurred in the dimly-lit environment of Experiment 1 did not occur in the brightly-lit environment of Experiment 2.

There was a main effect of drug condition in both experiments. The finding that the environment modulated withdrawal behavior, particularly in males, suggests that the environment is an important variable to consider when assessing withdrawal symptoms. In Experiment 1, the animals were observed in a dimly-lit room in standard sized cages with bedding. The observation environment was constructed to model the home cage as closely as possible, with the intention of maximizing the rats' comfort. This environment contrasted sharply with the observation environment in Experiment 2, in which rats were observed in a brightly-lit room in cages without bedding. Because bright lights can be stressful to rats (e.g., Frye and Orecki, 2002; Slawecki, 2005), it may be that the environment in Experiment 2 provided a stressor for the rats and may have potentiated the behavioral effects of nicotine withdrawal. In addition to the bright lights, it is also likely that the larger cage size and absence of cage bedding in Experiment 2 contributed to the differences in

Table 1
Nicotine/saline withdrawal behavior ratios

	Experiment 1 dimly-lit environment		Experiment 2 brightly-lit environment		
	WD1	WD2	WD1	WD2	WD3
Males	1.68	1.34	1.98	1.51	1.27
Females	1.49	1.36	2.17	1.49	1.26

Total withdrawal symptom behaviors of rats that had received nicotine divided by total withdrawal symptom behaviors of rats that had received saline for each sex within each observation session.

withdrawal behavior observed between the two environments by making the observation environment even more unlike the home cage.

It is noteworthy that some withdrawal behaviors are subtle (e.g., ptosis, empty-mouth chewing) and easier to observe in a brightly-lit environment. While some withdrawal behaviors were observed more frequently in the brightly-lit environment (e.g. empty-mouth chewing, whole body shakes), some behaviors were observed with similar frequencies in both environments (e.g., "other" behaviors), and some behaviors were observed more frequently in the dimly-lit environment (e.g., abnormal grooming). This pattern of observations does not support the interpretation that environmental differences in withdrawal behavior resulted from a compromised ability to see the behaviors. However, to ensure that reported environmental differences did not result from limited visibility, a more conservative analysis was conducted on the most easily observable behaviors (whole body shakes, abnormal grooming, abnormal posture or movement, and "other" behaviors). The reported effects remained significant even when analyzed more conservatively, supporting the interpretation that environmental differences did not result from compromised visibility.

Behavioral signs associated with withdrawal were increased in all female rats after cessation of drug administration, including in those that had received saline. It is likely that many of these behaviors resulted from discomfort after surgery to remove the pump. Despite the fact that signs of withdrawal were increased in all females, it is clear that many of these behaviors resulted from nicotine withdrawal, because significantly more behavioral signs of withdrawal occurred in female rats that had received nicotine than in those that had received saline. The withdrawal behavior ratios included the total amount of signs of withdrawal displayed by females that had received saline in the denominator to account for effects of pump-removal surgery discomfort in the expression of behavioral signs of nicotine withdrawal. Interestingly, female control rats had more behavioral signs of withdrawal in the dimly-lit environment than in the brightly-lit environment. It is not possible to compare the female controls statistically because they were from different experiments.

Rats were observed at four time points in Experiment 1 and five time points in Experiment 2. There was no concern that repeated exposure to observation cages would affect the subjects' behavior in Experiment 1 because the observation environment was intended to be similar to the familiar home-cage environment. However, the observation cages in Experiment 2 were intended to be dissimilar from the home-cages. It is possible that repeated exposure to the observation environment in Experiment 2 may have decreased the unfamiliarity of the environment somewhat by the time the last observations were conducted.

The reported increase in behavioral signs of withdrawal in rats following cessation of chronic nicotine is consistent with previous nicotine withdrawal research (e.g., Malin et al., 1993, 1994, 1996, 1998, 2001; Carboni et al., 2000; Epping-Jordan et al., 1998; Hildebrand et al., 1997, 1998; Watkins et al., 2000; Skjei and Markou, 2003; O'Dell et al., 2004; Phillips et al., 2004). While previous nicotine withdrawal research did not specify which behavioral signs of withdrawal differed significantly by drug condition, the O'Dell group (2004) reported effects of drug condition on eyeblinks in adult male rats. The present finding of an effect of drug condition on eyeblinks, as well as on total withdrawal behaviors, is consistent with the results of O'Dell et al. (2004). The reported experiments are the first to examine nicotine withdrawal in adult male and female rats.

4.1. Estrous measurements

The fact that estrous cycle phase did not influence withdrawal behavior is consistent with the research of Donny et al. (2000), who reported that estrous cycle phase had no effect on nicotine self-administration. Interestingly, estrous cycle phase has been found to influence self-administration of other drugs, including heroin, cocaine, and ethanol (e.g., Lynch et al., 2002, Roberts et al., 1998). Future research examining the effect of direct hormonal manipulations on nicotine withdrawal and self-administration is needed.

4.2. Potential limitations

Because rats are nocturnal animals that naturally avoid the light, exposure to bright light is a stressor for rats, and has been used as a stressor in experimental investigations (e.g., Frye and Orecki, 2002; Slawecki, 2005). However, no biological or behavioral assessments of stress were made in the present experiments.

It is possible that the estrous procedure was a chronic mild stressor for the females despite the efforts made to minimize discomfort during estrous collection. Estrous samples were also collected in a dark room illuminated only by a red light to further minimize undue stress. If the estrous collection did constitute a chronic stressor for the females, then it could potentially explain why the saline females demonstrated a greater magnitude of withdrawal behaviors than the saline and nicotine males in Experiment 1. However, the saline females demonstrated a comparable level of withdrawal behaviors to the saline males during the baseline and drug observation days.

In addition, male and female rats metabolize nicotine differently, with female rats having a reduced rate of nicotine metabolism and a larger volume of distribution of nicotine when compared to male rats (Kyermaten et al., 1988). Rats were observed during the optimal time period for observing nicotine withdrawal, as reported by Malin et al. (1992). However, identification of this optimal time period was based on observations of male rats. The reported sex differences in metabolism and distribution raise the possibility that the optimal time period for observing nicotine withdrawal in females may differ from the optimal time for observing withdrawal behaviors in males. This is a limitation of the current research. However, the fact that all rats were observed at multiple time points after nicotine cessation may have mitigated this limitation.

Lastly, length of the behavioral observation period differed in the two experiments. The observation period in Experiment 2 was 5 min longer than the observation period in Experiment 1, which likely contributed to the total amounts of withdrawal behavior observed. Withdrawal behavior ratios were calculated to account for this difference. However, the rats may have been acclimating to the observation cages during the first 5 min of the observation period, so the ratios may not be sufficient to fully address the difference. This limitation should be considered when interpreting environmental differences in withdrawal behavior.

4.3. Summary and implications

Cigarette smoking is a major public health concern, with approximately one-fifth of U.S. adults smoking cigarettes (CDC, 2007). Cigarette smoking prevalence is similar between men and women in developed countries, but women are less successful than men at quitting smoking (e.g., Perkins, 2001). The present experiment was conducted to determine whether reported sex differences in withdrawal (e.g., Shiffman, 1979), which are relevant to tobacco use and treatment, may be based on biological sex differences per se or on environmental influences.

There were three major findings in the present experiment. First, nicotine withdrawal exists and can be modeled in rodents, consistent with the work of Kota et al. (2007, 2008), Malin et al. (1992, 1994, 2006), O'Dell et al. (2004, 2006), and Phillips et al. (2004). Second, nicotine withdrawal exists in male and female rats. Third, environment modulates the expression of nicotine withdrawal behaviors, particularly in males. Males that had received nicotine displayed a greater amount of withdrawal behaviors in a brightly-lit environment than in a dimly-lit environment, while females that had received

nicotine displayed similar amounts of withdrawal behavior in both environments. The effect of the environment on withdrawal behavior was greater in male rats.

Adult male and female rats expressed nicotine withdrawal behavior after cessation of continuously administered nicotine. Differences between the saline and nicotine groups in withdrawal behaviors did not result from differences in overall locomotor activity, and female withdrawal behavior was not significantly affected by estrous cycle phase.

There were differences in withdrawal behaviors when rats were observed in different environments. There was a greater effect of environment on withdrawal behaviors in males. Males had more withdrawal behaviors in the brightly-lit environment than in the dimly-lit environment, but females had the same amount of withdrawal behaviors in both environments. In the dimly-lit environment, females had significantly more withdrawal behaviors than males, but in the brightly-lit environment, males and females that had received nicotine had similar amounts of withdrawal behaviors. The finding that female rats had substantial withdrawal behavior regardless of the environment in which they were tested is consistent with reports of greater sensitivity to effects of nicotine in females than in males (e.g., Grunberg et al., 1986; Harrod et al., 2004), that is, nicotine withdrawal in female rats is marked regardless of environment. In contrast, Kota et al. (2007, 2008) reported that male mice exhibit greater nicotine-induced behavioral withdrawal than do female mice.

4.4. Future directions

Recently, George et al. (2007) reported that nicotine withdrawal recruits the corticotropin-releasing factor (CRF) system and activates CRF_1 receptors which results in anxiety-like behaviors. Additional stressors during withdrawal may augment the CRF–CRF_1 system and exacerbate withdrawal. In the present research, nicotine withdrawal may have been affected by environmental stress, especially for male rats. If the present findings and the research of George et al. (2007) generalize to humans, then pharmaceutical therapies for smoking cessation that target the CRF–CRF_1 system should be developed (Grunberg, 2007). Whether such pharmaceuticals should be altered (in delivery rates, dosages, or other ways) based on sex is yet to be determined.

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