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Sex differences in attenuation of nicotine reinstatement after individual and combined treatments of progesterone and varenicline

Natashia Swalve, Ph.D.^{*}, John R. Smethells, Ph.D., and Marilyn E. Carroll, Ph.D.

Department of Psychiatry, University of Minnesota, Minneapolis, MN, 55455, USA

Abstract

Tobacco use is the largest cause of preventable mortality in the western world. Even after treatment, relapse rates for tobacco are high, and more effective pharmacological treatments are needed. Progesterone (PRO), a female hormone used in contraceptives, reduces stimulant use but its effects on tobacco addiction are unknown. Varenicline (VAR) is a commonly used medication that reduces tobacco use. The present study examined sex differences in the individual vs. combined effects of PRO and VAR on reinstatement of nicotine-seeking behavior in a rat model of relapse. Adult female and male Wistar rats self-administered nicotine (NIC, 0.03 mg/kg/infusion) for 14 days followed by 21 days of extinction when no cues or drug were present. Rats were then divided into 4 treatment groups: control (VEH+SAL), PRO alone (PRO+SAL), VAR alone (VEH+VAR) and the combination (PRO+VAR). Reinstatement of nicotine-seeking behavior induced by priming injections of NIC or caffeine (CAF), presentation of cues (CUES), and the combination of drugs and cues (e.g. NIC+CUES, CAF+CUES) was tested after extinction. Male and female rats did not differ in self-administration of nicotine or extinction responding, and both showed elevated levels of responding to the CAF+CUES condition. However, males, but not females, reinstated active lever-pressing to the NIC+CUES condition, and that was attenuated by both VAR and VAR+PRO treatment. Thus, males were more sensitive to NIC+CUE-induced reinstatement than females, and VAR alone and VAR combined with PRO effectively reduced nicotine relapse.

Keywords

Nicotine; Self-Administration; Reinstatement; Sex Differences

1. Introduction

Tobacco use is the single largest preventable cause of mortality in the western world [1], and it is characterized by abstinence periods followed by relapse [2]. A significant challenge in treating tobacco addiction is preventing relapse as only about 5% of smokers remain

^{*}Corresponding author: Natashia Swalve, Ph. D., University of Minnesota, 680A Diehl Hall, 550 Essex Street SE, Minneapolis, MN 55455, 612-624-8432, ; Email: nlswalve9220@gmail.com

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abstinent without treatment [2,3]. With treatment, abstinence rates still remain low with only 10–30% of smokers remaining tobacco-free long-term [4]. Since permanent tobacco cessation is necessary to limit the negative health consequences of tobacco use, more effective treatments to reduce relapse and maintain long-term cessation are necessary.

Progesterone (PRO) has shown promise in reducing smoking-related behavior. When endogenous PRO levels were high, motivation for nicotine (NIC), the primary psychoactive component of tobacco, was decreased in humans [5], nonhuman primates [6] and rats [7]. Conversely, in studies where endogenous PRO levels were low, rats showed greater motivation for NIC [8]. Increased plasma levels of PRO were associated with a 23% increase in the incidence of abstinence for each additional week of treatment in humans [9]. Exogenously-administered PRO also decreased self-administration and reinstatement (e.g. relapse) to cocaine, another commonly abused stimulant [10,11,12,13,14]. However, the effects of exogenous PRO on relapse to NIC use have not yet been examined.

Varenicline (VAR) has also shown efficacy for treatment of tobacco use. Varenicline is an $\alpha 4\beta 2$ nicotinic receptor partial agonist and $\alpha 7$ nicotinic receptor full agonist that is currently approved by the Food and Drug Administration for smoking cessation [15]. Varenicline has little to no abuse liability [16], and it is considered a safe and effective treatment for tobacco addiction [17]. Varenicline doubled to tripled successful tobacco cessation attempts compared to no treatment [18]. In rats, VAR also decreased the reinforcing effects of NIC, shown by attenuation of NIC self-administration and lower breakpoints for NIC on a progressive-ratio (PR) schedule [19,20]. Varenicline also blocked reinstatement of drug-seeking induced by NIC [21] and cues previously associated with NIC [19,22], supporting clinical findings of VAR's ability to reduce relapse to smoking.

Recent studies have shown that combination therapies may be a more effective method for increasing abstinence rates than individual treatments [23,24]. Combination therapies were associated with higher levels of abstinence compared with monotherapies [25,26,27,28,29], even with treatments from different pharmacological classes such as varenicline and bupropion [30,4]. Importantly, combination therapies did not increase risk to patients, as adverse reaction rates were similar between the individual and combined therapies [23]. Thus, combined treatments are a promising and safe method for reducing relapse to NIC use. Since both PRO and VAR were effective when used as individual treatments, the goal of the present research was to combine therapies in a nicotine relapse model in rats. Previous work had shown that the combination of PRO and another treatment, atomoxetine, was more beneficial than the individual treatments in reducing cocaine-seeking [31]. Similarly, the combination of a behavioral intervention (e.g. wheel-running) and PRO showed additive effects [32]. The combination of VAR with treatments such as NIC replacement [23] and bupropion [30,33] in humans has also been more successful than either treatment alone, similar to preclinical findings [34].

Finally, since males and females show unique patterns of relapse to tobacco addiction [35] as well as differential sensitivity to treatments for nicotine addiction, a goal of the present study was to compare effects of single and combined treatments in male and female rats. With other stimulants such as cocaine, females showed enhanced responsivity to

reinstatement of cocaine-seeking compared to males [11,36,37,38]. However, there are limited preclinical data on sex differences in nicotine reinstatement. In the one study on the topic, no sex differences in reinstatement were found to nicotine, cues, a pharmacological stressor or the combination of drugs+cues [39]. Finally, both VAR and PRO have shown sex-specific treatment effects for attenuating NIC and other forms of stimulant use [11,32,41,42], with females showing better treatment effects than males. Thus, sex differences on overall levels of reinstatement, as well as potential sex-specific effects on the interaction between treatment and nicotine-seeking, need to be considered.

Not only is there limited research on sex differences in reinstatement of NIC-seeking to NIC with or without the addition of cues, sex differences in reinstatement to other drugs such as caffeine (CAF) have not been explored. Caffeine (CAF) produced robust reinstatement to NIC-seeking, and, similar to NIC, this effect was enhanced by the addition of CUES [40]. Reinstatement to CAF and CAF+CUES has only been tested in males; thus, it is not known whether there are sex-specific effects of CAF on reinstatement. In our recent work, a CAF priming injection enhanced reinstatement to cocaine-seeking behavior in females but not males [65]; however, it is unknown whether this enhanced sensitivity to CAF would transfer to NIC-seeking as well. Thus, one objective of the present research was to test sex differences in reinstatement of nicotine-seeking behavior to a CAF priming condition.

The overall goals of the present study were twofold: 1) determine sex differences in an animal model of nicotine relapse (e.g. reinstatement) and 2) compare males and females on individual and combined effects of PRO and VAR on nicotine-seeking during reinstatement generated by NIC, CAF, CUES, and their combinations. Females showed greater reinstatement to drug and cue-induced reinstatement to other stimulants [e.g. cocaine; 11,36,37,38] than males; thus, it was hypothesized that females would be more sensitive than males to all types of reinstatement (e.g. CUES, NIC, CAF, NIC+CUES, and CAF +CUES). Females also show enhanced treatment effects for stimulant use [11,32,41,42]; thus greater effects of both individual and combined treatments were hypothesized to occur in females compared with males.

2. Methods

2.1 Animals

Forty-seven female and 48 male drug-naïve Wistar rats (weighing 200–224 g for females and 250–274 g for males on arrival) were used as subjects. The age of the rats was between 63–77 days upon arrival from Harlan Sprague-Dawley Inc. (Madison, WI), with females and males matched for age. Acquisition and maintenance data from a subset of rats (approximately half of the males and females) were analyzed for acquisition differences and published as part of a separate study [43]. Upon arrival, all rats were pair-housed in plastic cages and habituated to the laboratory for at least 3 days with *ad libitum* access to food (Teklad 2018, Harlan Laboratories, Madison, WI) and water prior to beginning experiments. Food was then restricted to 20 g (male) and 16 g (female) that has been shown to maintain rats at 85% free-feeding weight, and water was available *ad libitum*. Animal health was checked daily, and rats were weighed weekly. Rats were tested in operant conditioning chambers at the same time each day in batches from 0900-1300 h, with food given at 1515

h. Daily sessions took place during the light phase of the light/dark cycle (lights on from 0600-1800h), with rooms maintained at 40–60% humidity and temperatures ranging from 22–23° C. All experiments were approved by the Institutional Animal Care and Use Committee (Protocol #1307-30762A) and were conducted in accordance with Principles of Laboratory Animal Care [44].

2.2 Apparatus

At the beginning of the experiments, rats were individually housed in hanging stainless steel cages and transferred to the same octagonal operant conditioning chamber for each daily session. This procedure was previously described by Anker et al. [45]. Experimental chambers were housed in sound-attenuating custom-built enclosures with a ventilation fan to provide background noise and airflow. Each individual chamber contained two levers located on opposing sides of the chamber with a stimulus light (4.76 W) above each lever. A house light (4.76 W) was located in the upper corner of the chamber. A syringe pump (PHM-100, Med Associates, St. Albans, VT) was attached to a swivel tether system (375/22PS, Instech, Plymouth Meeting, PA; C313CS-MN, Plastics One, Roanoke, VA) to deliver NIC infusions. The tether attached to the rat using a spring-covered harness (CIH95AB, Instech). Data were collected and stored on PCs running MED-PC IV (v. 4.3) software.

2.3 Drugs

(–) Nicotine tartrate salt (Sigma, St. Louis, MO) was dissolved in sterile saline and adjusted to a PH of 7.0 ± 0.2 with a dilute NaOH solution with additional heparin (5 USP/mL) added to limit patency loss. Nicotine was diluted to 0.36 mg/ml in sterile saline, with a final dose of 0.03 mg/kg that was administered via infusion. The infusion rate was 0.025 ml/s, with the length of the infusion adjusted for the weight of the rat (0.33 s/100g). Progesterone (Sigma Aldrich, St. Louis, MO) was dissolved in peanut oil to a dose of 0.5 mg/kg, and peanut oil served as a vehicle (VEH) for PRO. Varenicline was dissolved in sterile saline to a dose of 1 mg/kg, with saline used as a vehicle (SAL). The doses of PRO and VAR were based on previous work showing that these doses significantly attenuated drug-seeking behaviors and reinstatement responding after multiple priming conditions in rats [19,32,34,46,47]. Progesterone, VAR and their corresponding vehicles were administered subcutaneously (SC) 30 min prior to the self-administration session. Priming injections of NIC (0.15 mg/kg) and CAF (5 mg/kg) were administered intraperitoneally (IP) at session onset during the reinstatement condition. These doses were chosen based on literature showing reliable reinstatement of drug-seeking by NIC and CAF (in combination with cues) at these doses [40,48].

2.4 Self-administration surgeries

Rats were initially anesthetized for surgery with ketamine (60 mg/kg, IP) and xylazine (10 mg/kg, IP) until a toe pinch did not produce movement. Atropine (0.04 mg/kg) was given subcutaneously (SC) as a respiration aide. Once anesthetized, a chronic silastic catheter (single-beaded, Plastics One, Roanoke, VA) was implanted, leading from the right jugular vein to the right atrium and finally directed subcutaneously to a medial incision in the back of the neck. The distal end of the catheter was attached to a harness connected to a swivel-

tether system at the top of the cage exiting to a syringe pump to deliver infusions. Rats were given extended-release buprenorphine (1.2 mg/kg, SC; ZooPharm, Windsor, CO) and had ibuprofen added to the drinking water for post-surgery pain relief. Rats were allowed three days of recovery, with heparin (10 IU/kg) and baytril (10 mg/kg) flushed through their catheters daily. Weekly checks with a ketamine (10 mg/kg) and midazolam (0.5 mg/kg) mixture in sterile saline determined continuing patency, with patency defined as a loss of a righting reflex within 10 sec after infusion of the mixture. If a rat's catheter was no longer patent, a second catheter was implanted in the left jugular vein.

2.5 Behavioral Procedures

2.5.1 Acquisition—Acquisition of NIC self-administration began after recovery from surgery with no prior food training. Rats were placed into the operant conditioning chambers for daily 1-h sessions. At the start of the session, a house light was illuminated. They were first trained to press on the active (right) lever on a fixed-ratio 1 (FR 1) schedule for an infusion of NIC (0.03 mg/kg/infusion) and illumination of the stimulus lights above that lever. The FR1 schedule was used to allow for comparison between studies with other drugs in our laboratory [31,49,32,50], as well as our previous work with nicotine [51]. A 20-second timeout followed each active lever response, during which stimulus lights were turned off and responses had no consequence but were recorded. The inactive (left) lever produced no consequence but inactive lever presses were recorded as a measure of general activity. At the beginning of each acquisition session, ground food was placed on the active lever until stable responding (e.g. two days of responding with 8 infusions per session) was met. Rats were then allowed to self-administer NIC until the criteria were met (e.g. 5+ infusions during each session without ground food on the active lever). Subsequently, rats were moved to the maintenance phase.

2.5.2 Maintenance—The maintenance phase consisted of 10–14 sessions of stable responding for NIC. The criterion for a maintenance session was 5 or more infusions of NIC per 1-hr session. Rats had to complete at least 10 days of maintenance to be moved into the extinction phase, with a maximum of 14 days allowed. If rats failed to self-administer NIC, or were unable to meet the total maintenance session requirement before patency was lost, they were excluded from the experiment. Only the first 10 days of maintenance were used to examine group differences as not all rats completed the full 14 days.

2.5.3 Extinction—During extinction, all contingencies were removed from the active lever, but inactive and active lever presses were recorded. Extinction lasted 21 days, during which rats were divided into groups based on the treatment they were to receive during reinstatement. This procedure was identical to that used in our previous work with cocaine to ensure consistency between studies [31,49,32]. The four groups consisted of PRO treatment alone (PRO+SAL), VAR treatment alone (VEH+VAR), the combination PRO and VAR treatment (PRO+VAR) and the control group (VEH+SAL). Following extinction, rats were given initial treatment for 3 days before the reinstatement condition commenced.

2.5.4 Reinstatement—Reinstatement was induced by drug priming injections (e.g. NIC and CAF), stimulus light cues previously associated with the infusion (e.g. CUES), and drug

+cue conditions (e.g. NIC+CUES, CAF+CUES). All daily reinstatement sessions were conducted within-subjects in a counterbalanced order determined by the project's statistician. A single control session preceded each reinstatement session; for drug priming injections, these sessions consisted of saline injections while for cue presentations, these sessions consisted of no cues. Injections of PRO, VAR, or controls were given 30 min prior to the start of each reinstatement session.

2.6 Statistical Analysis

All data are expressed as mean $\pm$ SEM. The dependent measures during maintenance were total number of infusions and inactive lever presses, and during extinction and reinstatement they were number of active and inactive lever presses. A mixed-model two-way ANOVA was used to examine sex differences during maintenance and extinction with sex and session as the independent variables. To determine if priming conditions produced reinstatement of drug seeking, a mixed-model two-way ANOVA was conducted in the control group (sex and reinstatement type as independent variables). Reinstatement responding after each priming condition was analyzed using two-way repeated-measures ANOVA followed by post-hoc Tukey comparisons to compare individual groups. An alpha value of 0.05 was used to indicate significance. Statistical analyses were run using GraphPad Prism version 5.

3. Results

Figure 1 shows number of infusions over the first 10 days of maintenance. There was no main effect of sex or session and no interaction between sex and session on the total number of infusions ($p>0.05$). There was no significant main effect of sex or session and no interaction for inactive lever responding during maintenance (data not shown; $p>0.05$). The mean ratio of active to inactive lever-pressing was approximately 2:1 in both males and females (mean responses $\pm$ SEM over 10 sessions= 11.67 $\pm$ 3.21 for males and 11.61 $\pm$ 1.391 for females).

Figure 2 illustrates active lever responses during the 21 extinction sessions. No main effects of sex or session were found, and there was no significant interaction ($p>0.05$). There was no main effect of sex or interaction between sex and session on inactive lever responding during extinction ($p>0.05$), but there was a main effect of session [$F(1,20)=1.826$, $p<0.05$], (data not shown).

Figure 3 shows active lever responding in the male and female VEH+SAL groups over all 6 reinstatement conditions. There was a significant main effect of reinstatement condition on active lever presses [$F(5,110)=5.039$, $p<0.001$] and a sex X reinstatement interaction [$F(5,110)=2.718$, $p<0.05$], but there was no main effect of sex ($p>0.05$). Post-hoc Tukey analyses showed that males, but not females, had enhanced responding on the active levers in the NIC+CUES reinstatement condition in comparison to the SAL condition, and both males and females showed significant reinstatement responding in the CAF+CUES condition. There was a main effect of reinstatement type on inactive lever-pressing [$F(5,110)=5.242$, $p<0.001$], but there was no main effect of sex and no interaction. Post-hoc Tukey analyses showed that inactive lever-pressing was significantly higher during the CAF-

induced reinstatement session compared to the SAL condition for both males and females ($p < 0.05$).

Figure 4 illustrates responses during the NIC+CUES condition comparing males and females receiving different treatment combinations. Individual two-way ANOVA for each reinstatement condition showed no significant main effects of sex or treatment, and there were no significant sex X treatment interactions for reinstatement to SAL, NIC, CUES, CAF, or CAF+CUES ($p > 0.05$). There was a significant main effect of treatment [$F(3,87) = 4.074$, $p < 0.01$] and a significant interaction between sex and treatment [$F(3,87) = 4.361$, $p < 0.01$] on active lever responding, but there was no significant main effect of sex on the NIC+CUES reinstatement condition ($p > 0.05$). Post-hoc analyses showed that the VEH+VAR and PRO+VAR treatments attenuated reinstatement to NIC+CUES in males but not females. However, further analyses of inactive lever-responding did not show any main effects of sex or treatment nor a sex by treatment interaction (data not shown; $p > 0.05$). These results indicate that inactive lever-pressing, used here as a measure of general activity, did not contribute to the significant treatment effects that was found with active lever-pressing.

4. Discussion

Male and female rats had similar levels of nicotine self-administration during maintenance, and they did not differ in extinction responding. Both males and females showed significant reinstatement to the CAF+CUES condition; however, only males showed significant reinstatement induced by NIC+CUES, and neither males nor females reinstated to any other condition. Reinstatement responding during the NIC+CUES condition in males, but not females, was significantly decreased by VAR alone as well as the combination of PRO +VAR, but the combination did not reduce reinstatement to levels beyond the VAR only treatment. This suggests that VAR is an effective treatment for NIC+CUES-induced reinstatement in males but combining VAR and other novel therapeutics, such as PRO, may not enhance its efficacy in males or females.

Sex differences in NIC self-administration, extinction and reinstatement have been examined in relatively few studies. The present results showing that adult female and male rats took similar numbers of infusions during NIC self-administration, which is consistent with a number of previous reports [8,43,52,39,53,54]. While enhanced motivation and NIC intake in female rats has been reported in some studies [52,55,56], this typically occurred only when increased behavioral output was required. For example, females showed potentiated NIC self-administration only under higher FR values [55,56] or under progressive-ratio schedule requirements [52]. Thus, the results of the present study were consistent with previous findings that sex differences in NIC self-administration did not emerge until required effort to receive the drug is substantially increased.

The extinction phase, that represents drug-seeking behavior after access to nicotine has been terminated and abstinence from nicotine use in humans, was also characterized by similar levels of responding between male and female rats. This is contrary to findings of Feltenstein and See showing more resistance to extinction in female rats compared to males

[39]. However, methodological differences in extinction between Feltenstein et al. [39] and the present experiment could explain these discrepant findings. In the present study, all contingencies including visual stimuli were lifted during extinction; whereas, Feltenstein et al. [39] retained the visual stimuli attached to the active lever. Since females are more sensitive to environmental stimuli previously paired with NIC [57], the absence of visual stimuli during extinction may underlie the lack of a sex differences during this phase. Given that the visual stimuli were not extinguished, it would be expected that females would have exceeded males in the cue-primed reinstatement condition; but male and female rats did not differ in this condition in the present study. Thus, it is unlikely that the presence of visual stimuli led to the lack of sex differences. The mechanisms behind these discrepancies between studies, whether methodological or biological, are currently unknown, and further examination of conditions leading to enhanced resistance to extinction in female rats is warranted.

The lack of reinstatement to NIC, CUES and CAF could be due to the low initial levels of NIC self-administration. In the present study, rats self-administered approximately 12 infusions in the 1-hr session, which is an amount that is lower than that reported in other studies at that nicotine dose [39,58], but the number of infusions was similar to that found in other studies that used an identical dose and session length [21]. The absence of prior food-training and use of a low FR schedule may have led to the lack of reinstatement to all conditions except for NIC+CUES and CAF+CUES. Feltenstein et al. [39] used similar conditions (e.g. no prior food-training, food restriction; FR1), and they only found reinstatement to a stressor and a NIC+CUES condition, similar to the results of the present study.

The present finding of NIC+CUES-induced reinstatement in males but not females is the first example of sex differences in NIC reinstatement. Previous results showed similar NIC and NIC + CUES reinstatement in both males and females [39]. Females were more sensitive to NIC-associated stimuli [57]; thus, it was unexpected that males had potentiated responding to the NIC+CUES condition. Methodological differences between the present work and that of Feltenstein et al. [39], such as session length, may have been a factor involved in the discrepancies. Future research on variables that influence sex-specific reinstatement is needed. However, initial results from this study suggest that males may have been more sensitive to relapse induced by a combination of NIC and NIC-associated cues.

The robust reinstatement to the drug+cues conditions, but lack of reinstatement to the drug-priming condition in the present study was consistent with the literature. Other studies suggested that the NIC+CUES priming condition was more likely to produce reinstatement than NIC alone [59,60,61], and this may have been due to the ability of NIC to enhance the conditioned-reinforcing effects of associated stimuli [62]. Similar to the NIC+CUES condition, reinstatement occurred only when CAF was paired with CUES in both males and females. In the only previous study on this topic, Liu and Jernigan [40] showed that rats did not reinstate to CAF alone if there was no CAF pre-exposure, but they reinstated to the combination of CAF+CUES. One potential explanation is that there was a generalization between the effects of CAF and NIC, as reinstatement occurred with both conditions when combined with cues. This hypothesis is supported by findings showing that caffeine

produced nicotine-like discriminative stimulus effects in a substitution task [63]. The interaction between caffeine, nicotine and nicotine-associated cues on relapse to nicotine use is important to our understanding of factors that maintain nicotine and tobacco use in humans; however, further exploration is needed the given conditioned reinforcement-enhancement effects of both drugs and the high rate of NIC and CAF co-use [62,64].

Sex differences were also found in treatment effects in the present study. Both VAR alone and in combination with PRO significantly decreased reinstatement (e.g. relapse) to NIC. This effect was only found in males in the NIC+CUES condition, which was unexpected since females showed a better response to VAR in clinical studies on smoking cessation [41]. The lack of a VAR effect in females may have been due to the low baseline levels of reinstatement in the majority of conditions. However, there was no significant treatment effect during the CAF+CUES reinstatement condition in females, even though they had higher baseline reinstatement rates. Thus, it is possible that VAR would have similar effects in both male and female rats if higher baseline levels of reinstatement were present in both sexes.

The effects of the individual and combination treatments may be specific to the type of reinstatement, as individual treatments of PRO and VAR did not significantly attenuate reinstatement to CAF+CUES in either sex. While VAR had no effect in females, the attenuation of reinstatement in males suggests that VAR was a viable treatment for certain subtypes of NIC use, specifically those involving the combination of nicotine and drug-associated cues. Moreover, contrary to the hypothesis that combination treatments would be more beneficial than individual therapies, the addition of PRO to the VAR treatment did not significantly enhance the attenuation, and treatment with PRO alone produced no effect. This suggests that the combination treatment effects were primarily driven by the effect of VAR. These results support previous work showing that VAR might be a beneficial treatment for cue-induced reinstatement [19,22].

5. Conclusion

Overall, these findings indicate that males and females did not differ in responding for nicotine in maintenance or extinction of nicotine self-administration, but both sexes showed elevated reinstatement responding when exposed to CAF+CUES. Males were more sensitive to the NIC+CUES priming condition than females; thus they may be more vulnerable to relapse overall. Under VAR treatment, both alone and in combination with PRO, males showed a reduction in NIC-seeking than females during reinstatement conditions. This VAR treatment effect may reflect a potential sex difference in treatment efficacy; however, this may have been driven by lower baseline responding in females than males. Further examination on sex-specific efficacy of commonly used treatments for cessation of nicotine-seeking is warranted.

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Highlights

- Male and female rats had no differences during maintenance and extinction.
- Both males and females showed reinstatement to the CAF+CUES condition.
- Male rats, but not females, reinstated to the NIC+CUES condition.
- Varenicline alone attenuated NIC+CUES reinstatement in males.
- Combination varenicline and progesterone decreased NIC+CUES reinstatement in males.

Figure 1. Mean (+SEM) active lever presses over the first 10 days of the maintenance period in 1 h daily sessions. There was no significant difference between males and females.

Figure 2.
Mean (+SEM) active lever presses over the 21 days of extinction. There was no significant difference between males and females.

Figure 3.

Mean (+SEM) lever presses in the VEH-SAL groups during all 6 reinstatement conditions. The bars with asterisks represent differences in responding during the reinstatement sessions. * denotes $p < 0.05$. Males showed increased responding to the NIC+CUES and CAF+CUES conditions, while females showed increased responding to the CAF+CUES condition.

Figure 4. Mean (+SEM) lever presses during the NIC+CUES reinstatement session. The bar with an asterisk indicates a difference between treatment groups during the NIC+CUES reinstatement session. * denotes $p < 0.05$.