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Research Article

Effect of Sex and Estrous Cycle on Nicotine withdrawal Syndrome in the Rat

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Abstract

Introduction: Severity of withdrawal syndrome in women during smoking cessation has reportedly been influenced by menstrual phase. There are few studies of female rats with their four-day estrous cycle.

Methods: Histological examination determined the precise estrous phase at time of testing. The subjects were 14 qualifying female rats and 8 male rats, all five to six months old. Slides of vaginal fluid were examined for estrous phase-identifying cell types. Nicotine withdrawal was evaluated at either the proestrus phase (n = 7), a follicular portion of the cycle, and the metestrus phase (n = 7) a luteal portion. Rats were continuously infused with 9 mg/kg/day s.c. nicotine bitartrate. On the seventh day, each subject was challenged with 1 mg/kg of the nicotinic antagonist mecamylamine, a dose that precipitates a vigorous withdrawal syndrome only in nicotine-dependent rats. Subjects were observed over 30 min. on a standard checklist of somatically expressed withdrawal behaviors.

Results: Male rats displayed 26.00 ± 3.64 withdrawal signs (M ± SEM), while female rats in proestrus exhibited a similar 28.86 ± 3.39 sign. Female rats in metestrus displayed 41.57 ± 5.38 signs. One-way ANOVA revealed a significant difference among groups, $p = .039$. *Post hoc* comparisons revealed significant differences between metestrus and proestrus females, $p = .049$ and between metestrus females and males, $p = .016$, but not between proestrus females and males.

Conclusions: The results are consistent with reports of menstrual phase influence on withdrawal severity in smoking cessation, providing a laboratory model for studying this issue and its potential treatment.

Implications: In the large literature on nicotine withdrawal in the rat, there are relatively few studies on female rats and hardly any on the effects of the estrous cycle on physical dependence and withdrawal. The results are consistent with reports on the menstrual cycle affecting withdrawal severity in women undergoing smoking cessation, supporting the translational relevance of the rat nicotine dependence model. The methods utilized here expand the ability of rat physical dependence models to compare the sexes and the estrous phases in nicotine physical dependence and withdrawal.

Keywords: Estrous cycle; Follicular phase; Luteal phase; Nicotine withdrawal; Sex

Introduction

Women have been reported to have higher average difficulty than men in smoking cessation [1-4]. There are also some gender differences in effectiveness of some smoking cessation treatments [5-7]. Such gender differences might plausibly be affected by the estrous or menstrual cycle [8,9].

The estrous cycle is divided into four phases [10]. The proestrus and estrus phases constitute the follicular phase, distinguished by maturation and release of the ovum and peaks in serum estrogen and progesterone. The metestrus and diestrus phases feature uterine implantation of the ovum, facilitated by progesterone from the ovarian corpus luteum [10].

Meta-analyses [10,11] supported earlier suggestions [12-15] that women undergoing smoking cessation experience a more severe withdrawal syndrome in their luteal phase. It would be useful if this sort of phenomenon treatments could be produced in the animal laboratory. This would allow detailed study of its biological mechanisms and its modulation by experimental

treatments. The present study determined the effects of sex and estrous cycle on intensity of nicotine withdrawal syndrome in the rat. It was hypothesized that female rats in metestrus would display more withdrawal signs than females in proestrus. These two phases were chosen because of the contrast between peaks of estrogen and progesterone levels during proestrus and much lower levels of each during metestrus [16]. Another hypothesis was that withdrawal signs in male rats would differ more from metestrus females than from proestrus females.

Methods

Subjects included sixteen female Sprague-Dawley rats, 5 to 6 months old, housed in two large cages with eight each to synchronize their estrous cycles. Eight male Sprague-Dawley rats of the same age were housed together. Experimental procedures accorded with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Determination of Estrous Cycle Phase

Prior to nicotine infusion, vaginal smears were taken each morning for eight days, thereby completing collection of two four-day cycles [17]. Vaginal fluid was collected with a micropipette filled with 40 µL of isotonic saline inserted into the tip of the vagina, and flushed until cloudy. Vaginal fluid was placed on a glass slide, one drop of 0.5% methylene blue stain was added, and photomicroscopic images were taken (Figure 1). All female rats were determined to be in the same estrous cycle phase prior to nicotine infusion. During nicotine infusion, cycle phases were checked daily to ensure consistency of the rats' estrous cycle. Vaginal smears were again examined immediately following observations for precipitated withdrawal, ensuring that females were in the predicted estrous cycle phase during withdrawal observation. Two female rats were disqualified from the study because the expected estrous phase was not confirmed in one of the smears.

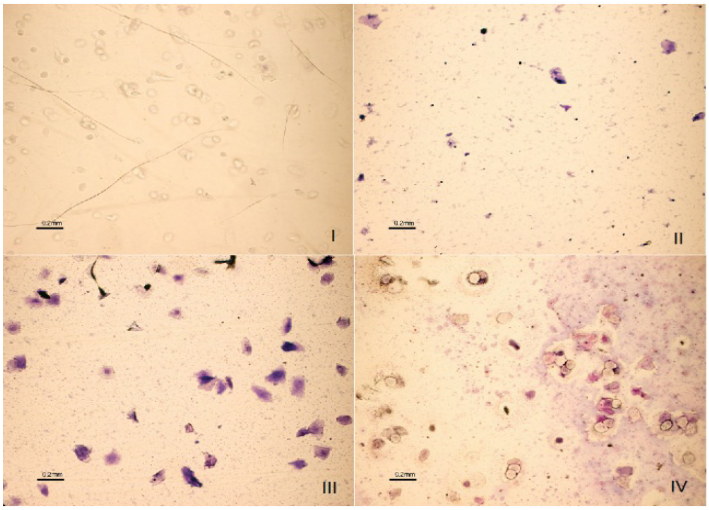

Figure 1: Vaginal smears (30x) indicative of four estrous phases in the rat.

(I) The proestrus phase of the estrous cycle is typically free of leukocytes and contains nucleated epithelial (round, nucleated) cells. (II) The estrus phase is typically free of leukocytes and contains cornified (irregularly shaped) epithelial cells. (III) The metestrus phase contains leukocytes (very small cells) and cornified epithelial cells. (IV) The diestrus phase contains leukocytes, like the metestrus phase. However, it can be differentiated from metestrus by the vacuolated (hollow) and nucleated epithelial cells characteristic of the phase.

Nicotine Dependence and Precipitated Withdrawal

A 2ML1 osmotic minipump was subcutaneously implanted in the shoulder region of each rat under isoflurane anesthesia. The pumps released nicotine bitartrate at a constant rate of 9mg/kg per day for seven days, resulting in similar nicotine blood concentration levels to those of heavy smokers [18].

There were three groups: eight male rats used as a comparison group, seven female rats implanted during their estrus phase so that they should be in proestrus at the end of infusion, and seven female rats implanted during the diestrus phase, so they should be in metestrus at the end of infusion. Examination of vaginal smears determined each rat's estrous phase immediately after observations.

To precipitate withdrawal, each rat was injected with 1mg/kg of mecamylamine, a noncompetitive nicotinic receptor antagonist [18]. This dose induces a withdrawal syndrome only in nicotine-dependent rats [19]. Thus, number of occurrences of withdrawal signs reflect the severity of nicotine dependence. Immediately after injection, rats were observed under "blind" conditions over 30 min. on a standard checklist of nicotine withdrawal behaviors (shakes/tremors, writhes/gasps, teeth chatters/vacuous chews, ptosis & miscellaneous less frequently observed signs: genital licks, hind foot scratches, backing up, and attempted escape jumps out of the observation chamber). Because tremors, chews and ptosis can sometimes continue for relatively long periods, continuous bouts of tremors and chews were not counted more often than once every 15 seconds, and ptosis was not counted more often than once every 60 seconds. Studies have supported the validity of overall numbers of these signs as an indicator of nicotine withdrawal severity [18,20].

Results

Males exhibited 26.00 ± 3.64 (M ± SEM) overall signs. Females in proestrus exhibited 28.86 ± 3.39 signs. In contrast, female rats in metestrus displayed 41.57 ± 5.38 signs. One-way ANOVA of overall withdrawal signs indicated a significant difference among the groups, $F(2,19) = 3.885$, $p = .039$. *Post hoc* analysis (Fischer's LSD test) revealed that females in the metestrus

phase exhibited significantly more total signs than females in the proestrus phase, p = .049, and than males, p = .016, which exhibited 26.44 ± 3.24 (M ± SEM) signs (Figure 2). The difference between females in the proestrus phase and males was not significant, p = .632.

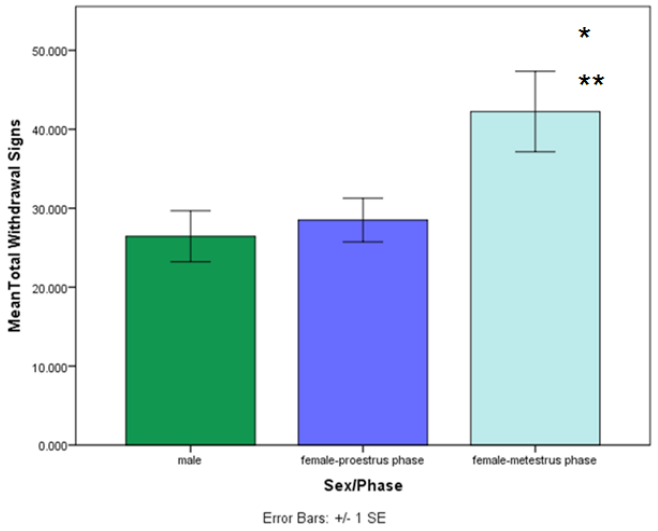

Figure 2: A one-way ANOVA indicated significance differences among groups in the number of total withdrawal signs exhibited. *p=.049 vs proestrus **p=.016 vs. males.

As shown in Table 1, female subjects in metestrus exhibited more signs than the other groups in all individual categories, except ptosis. *Post hoc* analysis (Fischer's LSD test) revealed that metestrus females exhibited significantly more vacuous chews than proestrus females, p = .025. Metestrus females had significantly more miscellaneous, less frequently observed signs than females in proestrus, p = .047, while their difference from males approached significance, p = .060. There were no other significant group differences in individual categories of withdrawal signs.

Sign	Male	Proestrus	Metestrus
Writhes/ Gasps	14.87 ± 2.84	19.43 ± 2.36	22.14 ± 4.52
Shakes/ Tremors	4.75 ± 1.49	4.43 ± 1.74	7.00 ± 1.98
Chews	3.37 ± 0.625	1.29 ± 0.68	4.86 ± 1.55[1]
Ptosis	1.88 ± 1.47	2.42 ± 0.78	1.0 ± 0.53
Miscellaneous	1.62 ± 0.65	1.14 ± 0.40	6.57 ± 3.09[2]
[1]p = .025 vs. proestrus, [2]p = .047 vs. proestrus and .06 vs. male (Fischer's LSD test).			

Table 1: Occurrences of Individual categories of withdrawal signs (M ± SEM).

Discussion

Previous studies with this model of rodent nicotine dependence have mostly been conducted with male rats. However, is increasingly clear that continuous subcutaneous infusion can induce considerable nicotine dependence in female Sprague Dawley rats. This was earlier shown by spontaneous withdrawal syndrome [21,22], and is now confirmed by antagonist-precipitated withdrawal in the present study. The results further suggest that females in the luteal phase, are more susceptible to severe nicotine withdrawal syndrome than females in the follicular phase and than males. The severity of withdrawal was not significantly different between male rats and females in the follicular phase. The results are consistent with clinical research on smoking cessation in women, suggesting more severe withdrawal in the luteal than the follicular phase [8,11,14,15]. Escape jumps are a withdrawal sign of particular interest because it has been commonly reported in morphine-abstinent mice [23] and not in nicotine-abstinent rats. Males showed absolutely no escape jump behavior, whereas females in the luteal phase had many occurrences of this severe withdrawal sign, suggestive of a panic reaction. Females in metestrus exhibited a mean of 3.71 escape jumps compared to 0.28 escape jumps shown by females in proestrus.

The four-day rat estrous cycle has led many researchers to avoid using female subjects for fear of increased behavioral variability. This sort of situation may create sex bias in the research literature, resulting in potential limitations of applicability to public health [24]. In situations where this is a concern, it can be dealt with by taking estrous cycle phases into account, as in the present experiment. If a research plan calls for making the two sexes more comparable, it may be advantageous to use females in the follicular phase. However, in preclinical drug research, it may be valuable to also study the luteal phase with its hormonal fluctuations and its potential influence on dependence and withdrawal syndrome [11].

The present data provide additional evidence for translational relevance of rodent nicotine dependence models [18-20] to phenomena observed in human smokers [11]. However, in addition to withdrawal severity, the human estrous cycle may affect other variables relevant to smoking cessation. For example, estrous phase may alter subjective response to and craving for nicotine [25,26].

The present results open up possibilities for future research to determine effects of estrous phase on additional measures of nicotine withdrawal severity, such as conditioned place aversion, intracerebral self-stimulation threshold or the anxiety response in

the elevated plus-maze. Further research directions might include assessing the other two phases (estrus and diestrus) within the follicular and luteal potions of the cycle. In addition, the effect of estrous phase at the onset of drug exposure could be studied for any influence on initial dependence formation. It would also be interesting to determine the effects of direct separate and combined progesterone or estradiol administration on nicotine dependence and withdrawal syndrome.

In conclusion, the animal laboratory results further confirm the interaction between sex and the estrous cycle in modulating nicotine dependence and withdrawal. The results also suggest a rapid and efficient laboratory model to study the biological basis and experimental treatment of this phenomenon.

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Contributors

MH and DM conceived the study. All authors participated in experiments, data collection, and analysis. All authors have also contributed to and approved the final manuscript.

Conflict of Interest

No authors had any conflict of interest.

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