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Sex differences in anxiety-like behavior and locomotor activity following chronic nicotine exposure in mice

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Abstract

Smoking appears to increase overall levels of stress, despite self-reports that men and women smoke to control symptoms of anxiety. The overall incidence of anxiety disorders is also significantly higher in women. This study examined whether behavioral sensitivity to chronic nicotine varies across sexes in mice. Male and female C57BL/6J mice were exposed chronically to nicotine in the drinking water (50, 100, or 200 µg/ml) and tested for locomotor activation and anxiety-like behavior in the elevated plus maze (EPM). Female mice were less sensitive to locomotor activation. Whereas both males and females showed increases in locomotor activity at the highest (200 µg/ml) concentration of nicotine, only males showed locomotor activation at the middle (100 µg/ml) concentration. The decreased sensitivity in females could not be explained by reduced nicotine intake compared to males. In the EPM, nicotine produced an anxiogenic-like response in females, but had no effect in males. Treatment with the high (200 µg/ml) dose of nicotine reduced the amount of time spent in the open arms of the EPM in female, but not male mice. No differences in the anxiogenic-like response to chronic nicotine was observed between $\beta 2$ -subunit knockout and wildtype mice, suggesting that $\beta 2$ -subunit containing nicotinic receptors do not mediate the anxiogenic-like response to chronic nicotine in females. This shows that female mice have an anxiogenic-like response to chronic nicotine, but are less sensitive to nicotine's psychostimulant properties, which may be related to the increased relapse to smoking following abstinence in women.

Keywords

C57BL/6J mice; nicotine; elevated plus maze; locomotor activity; nicotinic acetylcholine receptor; knockout mice

INTRODUCTION

Tobacco smoking is the leading preventable cause of death in the US [44]. Although men smoke more than women, the decline in smoking prevalence has been slower for women [38]. Women have less success quitting smoking, and are more likely to relapse, than men [2]. These factors put women at increased risk for smoking related illness and warrant the investigation of biologically-based sex differences in nicotine responses.

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Women may show reduced abstinence because nicotine replacement is less effective in women than men [23,45]. Studies by Perkins and colleagues suggest smoking behavior in women is reinforced less by nicotine intake and more by non-nicotine factors [33,34]. Negative mood also may play a greater role in smoking relapse for women [5].

In animal models, positive reinforcing effects of nicotine may be greater in female than male rodents. Females show faster acquisition of intravenous nicotine self-administration, higher break points on a progressive ratio schedule [11] and greater preference for nicotine in a two-bottle choice task [25,28]. Female rodents may self administer nicotine at higher rates because nicotine is more reinforcing, or because of differences in sensitivity. In mice, females were less sensitive to nicotine's locomotor depressant effects [18], but female rats showed increased sensitivity to nicotine's locomotor stimulant effects [4,14,17,20,39–41].

Nicotine has complex effects on anxiety, with both anxiolytic and anxiogenic effects of nicotine that vary depending on the behavioral model, nicotine dose, route or time course of administration (reviewed in [35]). With respect to sex, female rats were more sensitive to nicotine's anxiolytic effects in the social interaction test [8], but less sensitive in the elevated plus maze (EPM) [12].

Here, we explore sex differences in behavioral responses to chronic nicotine in mice on measures of sensitivity (locomotor activation) and anxiety (elevated plus maze). Previous studies have shown that $\beta 2$ -subunit containing nicotinic acetylcholine receptors ($\beta 2^*$ nAChRs, * represents other subunits) are essential for positive reinforcing [36] and locomotor activating effects [24] of nicotine. We therefore also determined what role $\beta 2^*$ nAChRs play in nicotine's effects on anxiety using the EPM.

MATERIAL AND METHODS

Animals

C57BL/6J (B6) mice were obtained from Jackson Laboratory (Bar Harbor, ME). $\beta 2$ subunit knockout mice ($\beta 2$ KO) were backcrossed 12–20 generations to B6 mice. Mice were housed 4–5 to a cage in a colony room maintained at 22°C on a 12 hour light-dark cycle (lights on: 7AM). All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Yale Animal Care and Use Committee.

Nicotine Treatment: Elevated Plus Maze and Locomotor Activation

The hydrogen bitartrate salt of nicotine (Sigma, St. Louis, MO) was used and concentrations were calculated as free-base. Mice were treated with 2% saccharin (Sigma) in 2mM tartaric acid (Sigma) or 50, 100, or 200 μ g/ml nicotine in 2% saccharin (Sigma). The pH of all solutions was adjusted to the nicotine solution. All solutions were administered in drinking water as the sole source of fluid at concentrations based on previous studies of drinking water administration in mice [24,43].

Elevated Plus Maze

The EPM was constructed of white Plexiglas and consisted of two open arms (5 mm lip) and two closed arms (black Plexiglas, 15 cm walls). Arms were 30.5 cm long and 5.5 cm wide. Maze was positioned 31.5 cm above the ground in dim lighting.

The EPM test was performed as described [27]. Group-housed female and male wildtype ($\beta 2$ WT) and $\beta 2$ subunit knockout mice ($\beta 2$ KO) were administered saccharin or 200 μ g/ml nicotine in drinking water for at least one month. Groups: SACCHARIN, male $\beta 2$ WT (n=8), male $\beta 2$ KO (n=9), female $\beta 2$ WT (n=11), female $\beta 2$ KO (n=10); NICOTINE, male $\beta 2$ WT (n=8),

male β 2KO (n=8), female β 2WT (n=14), female β 2KO (n=12). Mice were tested between 7AM and 9AM to assure nicotine was still on board during testing. Mice were placed at the intersection of the arms and allowed to explore for 5min. Two observers blind to genotype scored entries into the open and closed arms and time spent in open arms. Open arm entries were scored when all 4 paws crossed into the arm. Percent time spent in open arms (% time in open arms/time in open+closed arms) was used as a measure of anxiety-like behavior. Number of entries into the closed arms was used as a measure of locomotor activity.

Locomotor Activity

Group housed female and male mice were administered saccharin or nicotine (50, 100, or 200 μ g/ml) in drinking water for at least one month. Groups were SACCHARIN: females (n=17), males (n=15); 50 μ g/ml NICOTINE: females (n=17), males (n=15); 100 μ g/ml NICOTINE: females (n=15), males (n=14); 200 μ g/ml NICOTINE: females (n=16), males (n=15).

To measure locomotor activity, mice were singly housed in cages (19 $\times$ 29 $\times$ 13 cm) within the locomotor apparatus with food available at all times. Mice continued to drink nicotine or saccharin solutions for the duration of the test. The custom built locomotor apparatus (6 photocells, 4 cm apart, beam breaks collected in 1 hr blocks) was housed under the same lighting, temperature, and humidity conditions as the colony room. Mice acclimated to the cages housed within the apparatus for 1 day, and locomotor activity was recorded for 3 additional days.

Bottles were weighed and body weights were taken at the beginning and end of the experiment to compare nicotine intake between groups. To assess cotinine levels, blood was collected by decapitation between 7–8:30AM in mice that had nicotine in their drinking water.

Measurement of plasma cotinine levels

Blood was centrifuged, serum removed and stored at -80°C for later analysis. Serum nicotine and cotinine levels were measured by reversed-phase HPLC with ion pairing as described previously [15,16]. Internal standard was added, sample was alkalized, extracted with dichloromethane and hexane, and back-extracted into 0.2 ml of 0.2M phosphoric acid. The aqueous phase was chromatographed on a C6 column (Alltech Associates). 100–300 μ l of serum was collected for each mouse. Serum levels <300 μ l were brought to volume with human lipid-stripped plasma (Scantibodies Laboratory) for chemical detection. Cotinine concentrations were corrected according to the dilution factor.

Data Analysis

EPM: Percent time in open arms was calculated as a measure of anxiety-like behavior and the number of crosses into the closed arms was calculated as a measure of general activity. An analysis of variance (ANOVA) was performed on each dependent measure with DRUG (saccharin, 200 μ g/ml nicotine), GENOTYPE (β 2WT, β 2KO), and SEX (female, male) as independent variables.

Locomotor activity: number of beam breaks in 1 hr blocks was collected across the 24 hr circadian cycle starting at 7AM. The maximum one hour of activity (averaged over 3 days) between the hours of 5–8AM was used as the measure of locomotor activation because this activity peak is sensitive to nicotine in both males and females [24]. ANOVA was performed on the peak locomotor activity dependent measure with NICOTINE DOSE (0, 50, 100, 200 μ g/ml) and SEX (female, male) as independent variables.

Nicotine intake and cotinine levels: ANOVA was used with NICOTINE DOSE (0, 50, 100, 200µg/ml) and SEX (female, male) as a between subjects factor. Significant main effects and interactions were followed with the Tukey test.

RESULTS

Elevated Plus Maze

Effects of nicotine, sex, and $\beta 2^*$ nAChRs on anxiety-like behavior were examined using the EPM (Fig. 1). Chronic nicotine treatment was anxiogenic-like in the EPM in female, but not male $\beta 2$ WT and $\beta 2$ KO mice. No differences in closed arm entries were observed, consistent with the fact that nicotine-induced locomotor activation in mice is only seen when the animals are drinking non-stressfully in the home cage, and not in response to acute injection or movement into a novel environment

Locomotor Activity

Sex differences in sensitivity to the locomotor stimulatory properties of nicotine were evaluated in male and female mice treated chronically with 0, 50, 100, or 200µg/ml of nicotine in drinking water (Fig. 2). At the highest dose of nicotine (200µg/ml), locomotor activation was seen in both male and female mice. At the middle dose (100µg/ml), males, but not females, showed locomotor activation, suggesting that male mice are more sensitive to the stimulant properties of chronic nicotine. The lowest dose of nicotine (50µg/ml) did not produce locomotor stimulant effects in either male or female mice.

Nicotine Intake and Cotinine Levels

A subset of mice was tested to identify any sex-based differences in nicotine intake or plasma levels of nicotine. Both male and female mice show the expected increase in dose with increasing concentrations of nicotine (values in mg/kg/day for males: 50µg/ml, 7.8 ± 0.5 ; 100µg/ml, 14.3 ± 0.8 ; 200µg/ml, 28.3 ± 1.3 ; females: 50µg/ml, 10.8 ± 0.6 ; 100µg/ml 18.5 ± 1.2 ; 200µg/ml, 31.5 ± 1.3). There were no significant effects of sex or nicotine dose on mean plasma cotinine levels (values in ng/ml for males: 50µg/ml, 298 ± 50 ; 100µg/ml, 720 ± 109 ; 200µg/ml, 1289 ± 399 ; females: 50µg/ml, 575 ± 161 ; 100µg/ml, 489 ± 66 ; 200µg/ml, 611 ± 159). Males tended to show a dose-dependent increase in plasma cotinine (significantly higher plasma levels of cotinine in males at 200 vs. 50µg/ml). In contrast, female mice showed no relationship between nicotine dose and plasma cotinine.

DISCUSSION

This study demonstrates sex differences in C57BL/6J mice in response to chronic nicotine in measures of anxiety and locomotor activity. Chronic nicotine was anxiogenic in females in the EPM, but showed no effect in males. Females were also less sensitive to the locomotor stimulatory effects of nicotine, with locomotor activation exhibited only at the highest dose in females (200µg/ml) and at the middle and high (100, 200µg/ml) doses in males.

Chronic nicotine was anxiogenic in female mice, with decreases in time spent in open arms of the EPM in nicotine-treated females. Anxiogenic effects of chronic nicotine could not be explained by general decreases in locomotor activity, as measured by entries into the closed arms of the EPM. In contrast, males showed no effect of chronic nicotine in the EPM. Because males and females treated with the high dose of nicotine (200µg/ml) showed equivalent nicotine intake and plasma cotinine, sex differences in dose or metabolism of nicotine did not account for sex differences in behavioral responses. Both $\beta 2^*$ knockout and wildtype females showed similar reductions in the percent time in the open arms in the EPM, suggesting that these anxiogenic effects are not mediated by $\beta 2^*$ nAChRs. Finally, no overall differences in

anxiety-like behavior were noted between $\beta 2^*$ knockout and wildtype mice, confirming that $\beta 2^*$ receptors do not mediate baseline anxiety levels in mice [37].

Previous studies have shown that nicotine can be anxiolytic [6,30], anxiogenic [31], inactive, or can antagonize the anxiogenic effect of other drugs [21] in the EPM. Nicotine dose, length and timing of nicotine administration, strain, or baseline levels of anxiety have been well-characterized [35], few experiments have examined how sex may impact nicotine's effects on anxiety. In one study in adolescent rats, nicotine was anxiogenic in the EPM in females but anxiolytic in males [12]. Sex differences were also reported in the social interaction test, with adolescent female rats showing greater sensitivity to the anxiolytic actions of nicotine [8]. In adult rats, however, no sex differences were found in acute effects of nicotine in the EPM, and nicotine was anxiogenic in both males and females [12]. In contrast, in adult mice, acute nicotine had anxiolytic effects in the EPM, with females showing less sensitivity than males [9]. In the present experiment, mice treated with chronic nicotine showed a unique pattern of response, with females exhibiting an anxiogenic response, and males showing no effect. Future studies examining nicotine and anxiety should determine how sex interacts with the other variables described here.

The present study tested whether $\beta 2^*$ nAChRs contribute to chronic nicotine's effects on anxiety-like behavior. The magnitude of response to chronic nicotine was equivalent in female $\beta 2^*$ receptor knockout and wildtype mice, suggesting that $\beta 2^*$ receptors are not involved in anxiogenic-like effects of chronic nicotine in the EPM, although $\beta 2^*$ receptors may be involved in other aspects of anxiety. Promising candidates for mediating anxiety like behavior in the EPM are $\beta 4^*$ and $\beta 3^*$ subunits. Knockout mice lacking $\beta 4^*$ [42] or $\beta 3^*$ [3] subunits showed less anxiety-like behavior than wildtype mice in the EPM, suggesting that endogenous ACh may produce anxiogenic effects under conditions of stress mediated by $\beta 3^*$ and $\beta 4^*$ nAChRs.

Sex differences were observed in the psychostimulant effects of nicotine in response to chronic exposure. This finding expands on our previous report [24], showing that the high (200 μ g/ml) dose of chronic nicotine resulted in locomotor activation in both females and males. In the present study, only males showed activation at the middle dose (100 μ g/ml). It is unlikely that this difference in sensitivity can be explained by sex differences in nicotine intake or plasma levels of nicotine. At the 100 μ g/ml concentration, nicotine intake was actually higher in females than males, resulting in equivalent plasma levels of cotinine for both sexes. The current results differ from previous reports showing greater sensitivity in female rats to the locomotor stimulant effects of chronic nicotine [4,14,17,20]. Future studies will determine if the nature of these differences are due to species (mouse vs. rat) or route of administration (drinking water in the present study vs. iv administration [4,17,20] and minipump [14]).

Several potential mechanisms may explain sex differences in response to chronic nicotine. First, pharmacokinetic variations in plasma or brain levels of nicotine could explain sex differences. One study found that C57BL/6 females eliminated nicotine faster than males [18]. Studies in humans have found that women metabolize nicotine more rapidly, an effect related to estrogen [1]. Second, chronic nicotine differentially regulates nicotinic receptors in males and females, with males showing more pronounced nAChR upregulation than females [26,29]. Third, steroid hormones modulate responses to nicotine and both progesterone and estradiol blocked nicotine-induced analgesia in one study [9]. Estrogen also enhances dopamine release from striatal slices in females, but decreases dopamine release in males [10]. As nicotine-induced increased locomotor in the current model is dopamine-mediated [24], estrogen-induced modulation of dopamine release could mediate sex differences observed in this test.

It should be emphasized that sex differences in the present study do not reflect general increases or decreases in sensitivity for a particular sex, but rather different sensitivities dependent upon the behavioral measure. This is consistent with other reports. Females were more sensitive to nicotine in oral self-administration [25,28], intravenous self-administration [11], operant response to visual cues [7], and reductions in body weight and feeding [13]. Females were less sensitive than males to the effect of nicotine in analgesia [9] and cognition (active avoidance) [46] tests, and were less sensitive to the anxiogenic effects of nicotine during ethanol withdrawal [19].

The pattern of behavioral response in the current mouse model, with females showing greater anxiety-like behavior but less sensitivity to dopamine-mediated locomotor activation, may represent an appropriate model of female smokers. It has been proposed that women are less sensitive than men to dopamine-dependent positive reinforcing effects of nicotine (see [34]). Furthermore, nicotine may contribute to negative mood states and anxiety in smokers (see [32]), an effect that may be more pronounced in women, who have higher rates of anxiety and depressive disorders than men [22]. Future investigation into the hormonal and neurochemical mechanisms underlying sex differences in behavioral responsiveness to nicotine will further aid in understanding the relationship between smoking and anxiety disorders.

In summary, female mice were less sensitive to the anxiogenic and locomotor activating effects of nicotine. These data suggest that non-nicotine therapeutics may be more useful for smoking cessation in women as compared to men. Future studies will determine whether similar sex differences are important for the lower cessation rates in women.

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Figure 1.

Nicotine has anxiogenic-like effects in the EPM in female C57BL/6J mice. Female (A,C) and male (B,D) mice were treated chronically with saccharin or nicotine (200 µg/ml) in their drinking water and mean (±SEM) %time spent in open arms (A,B) and number of entries into closed arms (C,D) are shown. Time in open arms ANOVA: significant drug x sex interaction ($F(1,72)=4.9$, $p<0.05$). Females treated with nicotine spent significantly less time in the open arms than females treated with saccharin (Tukey post hoc test). Number of entries into closed arms ANOVA: significant sex x drug interaction ($F(1,72)=4.5$, $p<0.05$). No differences in closed arm entries between females treated with saccharin or nicotine (Tukey post hoc test).

* $p<0.05$

Figure 2.

Male C57BL/6J mice are more sensitive to locomotor stimulatory effects of nicotine than female mice. Mice received saccharin, 50, 100, or 200 mg/ml nicotine chronically in their drinking water. Mean ($\pm$ SEM) beam breaks for female (A) and male (B) mice for one hour during the second burst of homecage activity is shown. The maximum hour of activity between 5–8am was averaged across 3 days. ANOVA: significant main effect of sex ($F(1,116)=17.5$, $p<0.0001$) and nicotine dose ($F(3,116)=5.8$, $p<0.001$). Female mice treated with 200 mg/ml nicotine were more active than the female saccharin control group. Male mice treated with both 100 and 200 mg/ml of nicotine were more active than male saccharin treated controls (* $p<0.05$ vs. vehicle, Tukey post hoc tests).