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Sex differences in the effects of nicotine on contextual fear extinction

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A B S T R A C T

Anxiety and stress disorders occur at a higher rate in women compared to men as well as in smokers in comparison to non-smoker population. Nicotine is known to impair fear extinction, which is altered in anxiety disorders. However, nicotine differentially affects fear learning in men and women, which may mean that sex and nicotine-product use can interact to also alter fear extinction. For this study, we examined sex differences in the effects of acute and chronic nicotine administration on fear memory extinction in male and female C57BL/6J mice. To study the acute effects of nicotine, animals trained in a background contextual fear conditioning paradigm were administered nicotine (0.09, 0.18 or 0.36 mg/kg) prior to extinction sessions. For chronic nicotine, animals continuously receiving nicotine (12.6, 18, or 24 mg/kg/day) were trained in a background contextual fear conditioning paradigm followed by fear extinction sessions. Males exhibited contextual fear extinction deficits following acute and chronic nicotine exposure. Females also exhibited extinction deficits, but only at the highest doses of acute nicotine (0.36 mg/kg) while chronic nicotine did not result in extinction deficits in female mice. These results suggest that sex mediates sensitivity to nicotine's effects on contextual fear memory extinction.

1. Introduction

Anxiety and stress disorders are a family of mental illnesses that include post-traumatic stress disorder (PTSD; American Psychiatric Association, 2013). Common among anxiety and stress disorder symptomatology is an exaggerated fear response to stimuli associated with an aversive experience (Parsons and Ressler, 2013; Rau et al., 2005). These fear-inducing associations can be weakened through exposure therapy in humans and with fear extinction in animals, which involves repeated exposure to the fear-inducing stimuli in the absence of danger (Milad and Quirk, 2012; Rauch et al., 2012). Importantly, nicotine use is bidirectionally associated with anxiety and stress disorders (Koenen et al., 2005; Morissette et al., 2007; Thorndike et al., 2006). Although nicotine product usage and anxiety disorders co-occur in both men and women, the strength of these associations differs between sexes (Breslau, 1995; Thorndike et al., 2006). PTSD and nicotine use are significantly associated only in men, but generalized anxiety and nicotine use are associated to the same extent in men and women (Breslau, 1995; Thorndike et al., 2006). Thus, sex may modulate the relationship between nicotine use and anxiety and stress disorders.

Conditioned fear extinction, which involves repeated exposures to a previously fear-associated cue until the fear response diminishes, is used as a model of exposure therapy in both animal and human studies. It has been shown that acute nicotine enhances contextual fear learning in male and female mice (Gould, 2003; Gould et al., 2004; Gould and Wehner, 1999). In male mice, acute nicotine has been shown to impair contextual fear extinction, stunting the normal decline of the fear response across extinction trials without affecting general freezing behavior (Kutlu and Gould, 2014; Kutlu et al., 2016a, 2017a, 2017b) and enhance spontaneous recovery of extinguished contextual fear (Kutlu et al., 2016b). Chronic nicotine has similar effects on contextual fear learning process. Results from our lab previously demonstrated that chronic nicotine delayed contextual fear extinction in male mice and this effect was sustained during withdrawal parallel to the period of nicotine-induced hippocampal nicotinic acetylcholine receptor upregulation (Kutlu et al., 2016c). However, sex differences in acute and chronic nicotine's effects on extinction of fear memory have not been explored. Therefore, in the present study, we examined sex differences in the effects of acute and chronic nicotine exposure on fear extinction.

2. Methods

Subjects were male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), 8–12 weeks of age. Animals were group-housed in a colony room maintained on a 12 h light/dark cycle (lights on at 9:00 am and lights off at 9:00 pm) with ad libitum access to food and water. Behavioral procedures occurred between 9:00 a.m. and 6:00 p.m. Experiments were conducted by individuals blind to

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conditions. All behavioral procedures were approved by the Temple University and Penn State University Institutional Animal Care and Use Committees. All behavioral procedures were performed at Temple University.

2.1. Apparatus

All behavioral procedures took place in one of four identical chambers (26.5 × 20.4 × 20.8 cm) housed in sound attenuated boxes. Chamber walls and ceilings were composed of clear Plexiglas and the floor was a grid of metal bars (0.20 cm diameter) spaced 1.0 cm apart and connected to a shock generator and scrambler (Med Associate, St. Albans, VT, model ENV-414). A speaker for CS presentation was mounted directly above the Plexiglas chamber in each box, and a ventilation fan attached to the side of the box provided background noise and air exchange. Each box was illuminated by a 4-watt light bulb mounted beside the speaker. Conditioned and unconditioned stimuli were controlled by a PC running LabView software (National Instruments, Austin, TX).

2.2. Drug administration

Nicotine hydrogen tartrate salt (Sigma, St. Louis, MO) was dissolved in 0.9% saline. For acute nicotine experiments, saline or nicotine (0.09, 0.18, and 0.36 mg/kg; freebase weights) was administered via intraperitoneal injection 2–4 min prior to behavioral procedures. The acute nicotine doses are chosen based on previous studies from our laboratory examining the effects of acute nicotine on fear extinction (Kutlu and Gould, 2014). For chronic nicotine experiments, nicotine was delivered via subcutaneous osmotic minipump (Alzet, Model 1002, Durect, Cupertino, CA), which allows continuous nicotine administration up to 14 days. Minipumps were surgically inserted through an incision made on the upper back of the mouse while mice were under 2–5% isoflurane anesthesia. Surgeries were conducted under aseptic conditions. Mice in the chronic nicotine experiment received continuous nicotine (12.6, 18, or 24 mg/kg/day; freebase weights). Control animals received sham surgeries, for which identical surgical procedures aside from pump implantation were performed. It should be noted that true blinding to drug conditions could not be achieved, as pumps were present during behavioral training for nicotine mice. As training began on the 7th day following minipump implantation or sham surgery, animals were allowed 6 full days to recover prior to behavioral training. All mini-pumps were validated following removal to ensure adequate amount of nicotine administration. Chronic nicotine doses are based on previous studies from our laboratory showing the impairing effects of chronic nicotine on contextual fear extinction in male mice (Kutlu et al., 2016c).

2.3. Behavioral procedures

Fear extinction procedures were based on previous reports examining the effects of acute nicotine on contextual fear extinction (Kutlu and Gould, 2014). Animals first underwent contextual fear conditioning training, wherein mice received two white noise-footshock (0.57 mA) pairings. All behavioral sessions were approximately 5 min in length. All freezing behavior was scored using a time sampling method, for which the experimenter live-scored mice (freezing: yes/no) every 10 s. Training sessions consisted of 2 min of free movement within the chamber to measure baseline freezing followed by 30 s of white noise. 0.57 mA shocks were 2 s in length, and co-terminated with white noise. Following termination of the first white noise-shock pairing, animals were scored for immediate freezing to shock for 2 min. After this, another 30 s of white noise with a co-terminating 2 s 0.57 mA shock. 24 h later, mice were returned to the training context for retention testing to determine initial freezing to the context. For contextual freezing, mice were placed back in the training context for 5 min in the absence of foot-shocks, and conditioned freezing was measured. Specifically, mice were simply allowed to move freely within the behavioral chambers for 5 min, and freezing behaviors were scored throughout. Beginning 24 h after the retention testing, all mice received single daily extinction sessions for 5 days. For each extinction session, mice were placed back in the training context for 5 min in the absence of foot-shocks and conditioned freezing was measured in the same manner as described above. For acute nicotine experiments, mice received drug 2–4 min before each extinction session. For the chronic nicotine experiments, training began on the 7th day following minipump implantation or sham surgery. Sample sizes for the acute nicotine were as follows: males $n = 7–17$, females $n = 9–25$. Disproportionate sample sizes between experimental and control groups resulted from the collapsing of saline animals run alongside all dose groups into one control group. Sample sizes for chronic nicotine were as follows: males $n = 8–10$, females $n = 9–10$.

2.4. Statistical analyses

For acute and chronic nicotine extinction, a 2-way ANOVA (drug × extinction day) was conducted on normalized freezing levels (freezing × 100 / initial freezing), wherein extinction day was treated as a repeated measure. Freezing was normalized to ensure differences in baseline freezing did not affect subsequent freezing curves (Tian et al., 2008; Kutlu et al., 2016b). Planned comparisons of extinction across doses were made using Bonferroni-corrected *t*-tests.

3. Results

3.1. Acute nicotine extinction

No significant Sex × Drug × Trial interaction ($F(15, 465) = 0.862$, $p = 0.60$) was found. Thus, Drug × Trial interaction was evaluated separately for each sex. For the acute nicotine experiment, animals underwent contextual fear conditioning and testing followed by 5 consecutive days of fear extinction. No baseline sex differences in initial freezing (Sex main effect: $F(1, 40) = 0.157$, $p = 0.694$) or in extinction (Sex × Trial interaction: $F(5, 200) = 1.135$, $p = 0.343$) were observed in saline treated animals. When administered before extinction sessions, acute nicotine impaired extinction in both males (Fig. 1a) and females (Fig. 1b). Separate 2-way ANOVAs yielded a significant interaction between Extinction Day and Drug in males ($F(15, 200) = 3.438$, $p < 0.001$) and in females ($F(15, 265) = 2.456$, $p = 0.002$). In addition, the Drug main effect was also significant for both males ($F(3, 40) = 6.654$, $p = 0.001$) and females ($F(3, 53) = 7.436$, $p < 0.001$). These results suggest that both males and females were affected by the nicotine treatment. However, Bonferroni-corrected *t*-tests showed that all doses of nicotine effectively impaired fear extinction in males (0.09 mg/kg: 4th day $p = 0.05$; 0.18 mg/kg: 3rd, 4th, and 5th days $p < 0.05$; 0.36 mg/kg: 3rd, 4th, and 5th days $p < 0.05$) whereas in females, only the 0.36 mg/kg dose impaired extinction (0.36 mg/kg: 2nd, 3rd, 5th days $p < 0.05$). Therefore, males may be more sensitive to the impairing effects of acute nicotine on fear extinction than females (see Fig. 1).

3.2. Chronic nicotine extinction

No significant Sex × Drug × Trial interaction ($F(15, 335) = 1.286$, $p = 0.23$) was found. Thus, Drug × Trial interaction was evaluated separately for each sex. For the chronic nicotine experiment, animals were administered nicotine for 14 days. Fear conditioning took place on the 7th day of chronic nicotine exposure, and the last day of fear extinction took place on the 14th day of chronic nicotine exposure. No baseline sex differences in initial freezing (Sex main effect: $F(1, 18) = 0.724$, $p = 0.406$) or in extinction (Sex × Trial interaction: $F(5, 90) = 0.861$, $p = 0.510$) were observed in sham surgery animals. Chronic