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Sexually diergic hypothalamic-pituitary-adrenal (HPA) responses to single-dose nicotine, continuous nicotine infusion, and nicotine withdrawal by mecamylamine in rats

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Abstract

Hypothalamic-pituitary-adrenal (HPA) responses to single-dose nicotine (NIC) are sexually diergic: Female rats have higher adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) responses than do males. In the present study we determined HPA responses in male and female rats following single doses of NIC, a single-dose of NIC immediately following continuous NIC for two weeks, and NIC withdrawal by single-dose mecamylamine (MEC) following continuous NIC infusion for two weeks. Blood sampling occurred before and after MEC and NIC administrations for determination of ACTH and CORT.

In accordance with our previous findings, female ACTH and CORT responses to single-dose NIC were greater than male responses. This sex difference remained after single-dose NIC followed continuous NIC infusion, but HPA responses in both sexes were significantly lower in magnitude and duration than in the single-dose NIC alone groups. Sex differences also were observed following NIC withdrawal by MEC: The HPA responses to pretreatment with MEC were significantly higher in magnitude and duration in the continuous NIC groups than in the single-dose NIC groups. These results demonstrate that HPA responses to NIC are reduced and transient following continuous NIC infusion but are enhanced and sustained following NIC withdrawal by MEC after continuous NIC, suggesting that NIC habituation and withdrawal influence the stress responses in a diergic manner. These findings highlight the importance of sex differences in the effect of NIC on HPA axis activity and stress responsiveness, which may have implications for directing NIC-addiction treatment specifically towards men and women.

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Keywords

ACTH; CORT; HPA axis; Mecamylamine; Nicotine; Sexual diergism

I. INTRODUCTION

Hypothalamic-pituitary-adrenal (HPA) axis activity in rats is reflected peripherally by plasma concentrations of the stress hormones adrenocorticotrophic hormone (ACTH) and corticosterone (CORT), released from the anterior pituitary and adrenal cortex, respectively. ACTH release is stimulated by corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) secreted from the paraventricular nuclei (PVN) of the hypothalamus [3,62]. HPA axis activity is modulated by cholinergic receptors in brain areas such as the PVN and supraoptic nuclei (SON) of the hypothalamus, the hippocampus, and the brainstem [19,32,41,59,74], as well as by cholinergic receptors within the axis itself [5,6,33].

Nicotinic cholinergic receptors in the brain are primarily presynaptic and enhance the γ -amino butyric acid, glutamate, norepinephrine (NE), and serotonin [49,73]. Nicotine (NIC) administration increases HPA axis activity in both humans [1,43,44,51,64] and rodents [17,18,27,41,46,52,57,78]. Because there are few reports of sex differences in HPA axis responses to NIC [2,24,46,55,57,63], our laboratory's focus has been to study sexual diergism (functional sex differences) in HPA axis responses to cholinergic stimulation [54,55,57,58,60]. We previously reported that NIC activated the HPA axis in a sexually diergic manner: NIC stimulated ACTH and CORT in female rats to a significantly greater degree than in male rats [46,55,57].

The behavioral and neurochemical effects of NIC also appear to differ between the sexes [23,50]. In both human and animal models, females self-administer NIC at lower initial doses than do males; they become addicted more quickly than males; and they are at greater risk of relapse following abstinence than are males [7]. Although several studies have determined the effects of NIC withdrawal in rodent models [14,21,22,29,31,69,75,76], few have determined the effects of NIC withdrawal on stress-responsive hormones of the HPA axis [2,63]. As well, to our knowledge no study has explored sex differences in HPA axis responses to single-dose NIC, NIC habituation, and NIC withdrawal in rodents. The present study therefore determined sexually diergic HPA axis responses to single-dose NIC, single-dose NIC immediately following continuous NIC administration for two weeks, and NIC withdrawal by single-dose MEC in male and female laboratory rats.

II. MATERIALS AND METHODS

Animals

Eight-week old, jugular vein-cannulated (JVC), male and female Sprague-Dawley rats weighing 200-225 g were obtained from Taconic Farms, Inc. (Germantown, NY, USA). Animals were housed singly in a well-ventilated, temperature- and humidity-controlled environment (22-25°C, 50-75% humidity) under a standard 12-h light/dark cycle (lights on at 0700 h). Laboratory rat chow and water were available *adlibitum*. The stage of estrous cycle in the females was uncontrolled and thus considered a random effect; we have shown the influence of estrous cycle stage on HPA activity to be of considerably less magnitude than the changes produced by NIC [55]. Prior to experimentation, animals were allowed 4-5 days to acclimate to the housing conditions and blood sampling via routine flushing of their cannulae. Experiments were performed between 0900 h and 1300 h to minimize circadian variations in plasma hormone concentrations. Twenty-three to 24 rats per sex were tested in the SAL groups; 15-20 rats per sex were tested in the single-dose NIC groups; and 15-18

rats per sex were tested in the continuous NIC administration groups. All experiments were approved by the Allegheny-Singer Research Institute Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Drug Administration

All doses of NIC (nicotine hydrogen tartrate salt; Sigma, St. Louis, MO, USA) were calculated and reported based on the weight of the free base. Immediately before each experiment, NIC was freshly prepared in SAL. On experiment days all single-doses of NIC or SAL were administered as IP injections after baseline blood samples were collected. To investigate the effects of single-dose NIC alone and following continuous NIC, animals were administered SAL (1 ml/kg) at -20 min, followed by SAL (1 ml/kg), or NIC (0.1, 0.3, or 0.5 mg/kg) dissolved in SAL, at 0 min; therefore, animals always received two injections. To investigate the effects of single-dose MEC alone and following continuous NIC, animals were administered MEC (mecamylamineHCl; 5 mg/kg; Sigma, St. Louis, MO) at -60 min, followed by SAL (1ml/kg) or NIC (0.3 mg/kg) dissolved in SAL at 0 min. Dosing time and concentrations of NIC and MEC were based on pharmacokinetic parameters and previous studies in rats [17,18,57,67,77], as supported by a recent review on NIC dose selection for *in vivo* studies [40].

Continuous NIC Administration

For the continuous NIC groups, Alzet® osmotic mini-pumps (model 2002) were surgically implanted into a 1 cm opening in the peritoneal cavity under pentobarbital (35-40 mg/kg) anesthesia. Before implantation, the mini-pumps were primed by injecting them with NIC (105 mg/ml) solution and placing them into 37°C physiological SAL for a minimum of 4 hours. Following insertion of the mini-pumps, the abdominal muscles were closed with 4-0 absorbable sutures in a non-interrupted pattern, and the skin was stapled with wound clips. Surgery time ranged between 10 and 15 min. Based on the initial NIC dose (105 mg/ml) and the osmotic rate of the model 2002 pumps, rats were delivered a continuous infusion of NIC at approximately 4.5 mg/kg/day (the approximate exposure of a smoker who uses one-half to one pack per day) for 14 days [31]. We did not measure plasma NIC concentrations, because the stability of NIC in the osmotic mini-pumps and the steady-state NIC concentrations in plasma following pump implantation are reliable and reproducible [11,12,31].

Blood Sampling

The two-person procedure for blood sampling from cannulated animals was used. One person gently contained the animal, which remained calm subsequent to daily handling, while the other person collected the blood sample. Each sampling was completed in less than 1 min. To maintain cannula patency, twice each week the stainless-steel cannula plug was removed, the heparin-polyvinylpyrrolidone (PVP) (100 IU/ml) lock solution (Sigma, St. Louis, MO) aspirated, and 0.1 ml buffered normal SAL injected, followed by replacement of 0.02 ml lock solution. A similar procedure was followed for blood sampling: The PVP lock solution was aspirated, and 300-325 µl blood was 1 ml withdrawn tuberculin syringe, immediately into transferred a into microcollection tubes, and stored on ice. A replacement solution of buffered normal SAL (37°C) equal to the amount of blood withdrawn was infused through the cannula, the cannula was injected with 0.02 ml lock solution, and the stainless-steel plug was reinserted. The plasma was separated by centrifugation, quickly frozen at -80°C, and stored until hormone analyses. Baseline blood samples were collected before and after administration of SAL or MEC. Four additional blood samples were collected 10, 20, 40, and 60 min after SAL or NIC.

Hormone Assays

Plasma ACTH was determined in singlet by a highly specific immunoradiometric assay (Nichols Institute, San Juan Capistrano, CA, USA & Diasorin, Stillwater, MN, USA). Inter- and intra-assay coefficients of variation were less than 8%, and the minimum detectable ACTH concentration was 1.7 pg/ml. Plasma CORT was determined in duplicate by radioimmunoassay (ICN Pharmaceuticals, Costa Mesa, CA). Antibody cross-reactivities were less than 0.5% for all other steroids. Inter- and intra-assay coefficients of variation were less than 10%, and the minimum detectable CORT concentration was 3.5 ng/ml.

Statistical Analysis

Group N's varied due to insufficient sample for analysis of both hormones from some of the animals, because of surgical complications and loss of cannula patency. Data are presented as mean $\pm$ standard error of the mean (SEM). Changes in plasma hormone concentrations following drug treatments were calculated for each animal as the post-drug change from its own baseline. Between-group comparisons of drug treatments used the SAL-treated group as control and were assessed by three-way (Sex $\times$ Drug $\times$ Time) analysis of variance (ANOVA), with time as a within-groups factor. Where appropriate, *post-hoc* pairwise comparisons were made with Tukey-Kramer tests to determine locations of significance. Significance was considered as $p < 0.05$. All data sufficiently approximated a Gaussian distribution such that no transformations were required (log-transformation did not alter the statistical results).

III. RESULTS

Behavioral Observations

NIC doses were chosen to minimize noxious side effects, because stress is a non-specific activator of the HPA axis [4]. Observable cholinergic effects such as increased defecation, urination, piloerection, salivation, tremor, and chromodachryorrhea ("red tears") were absent in all groups following NIC administration. Observable nicotinic effects such as sedation, flaccid posture, and loss of body tone, and observable anticholinergic behaviors such as agitation, teeth chatter, ataxia, eye blinks, and ptosis (facial/eyelid drooping) also were absent in all groups.

HPA Axis Hormone Responses

ACTH—Fig. 1 shows plasma ACTH concentrations (pg/ml) following SAL (Fig. 1A) and increasing doses of NIC (Fig. 1B-D). All groups were administered SAL at -20 min, followed by SAL (1 ml/kg) or NIC (0.1, 0.3, or 0.5 mg/kg) at 0 min. Baseline ACTH values were similar between sexes in all groups. NIC stimulated ACTH in both sexes in dose-response fashion, and females showed greater ACTH responses than males, as indicated by a highly significant sex $\times$ drug interaction ($F(3,145) = 9.9$; $p < 0.0001$), as well as by highly significant main effects of both drug ($F(3,145) = 77.2$; $p < 0.0001$) and sex ($F(1, 145) = 41.4$; $p < 0.0001$). Multiple comparisons at each time point between sexes, at each time point compared to baseline, and at each time point compared to SAL control are indicated in Fig. 1.

Fig. 2 shows plasma ACTH concentrations (pg/ml) following single-doses of SAL and NIC (0.3 mg/kg) administered after continuous NIC infusion and/or MEC pretreatment. NIC (0.3 mg/kg) was used as the single-dose challenge because it produced almost the same differential sex response as did NIC (0.5 mg/kg) (Fig. 1C,D). For the experiments in Fig. 2A-D, SAL was given at -20 min, followed by SAL (1 ml/kg) or NIC (0.3 mg/kg) at 0 min. For the experiments in Fig. 2E-H, MEC (5 mg/kg) was given at -60 min followed by SAL

(1 ml/kg) or NIC (0.3 mg/kg) at 0 min. For the experiments in Fig. 2B, D, F, and H, NIC was infused for two weeks via osmotic mini-pumps prior to single-dose SAL or NIC challenge. Note the expanded ordinate scale in Fig. 2E-H (0-250 pg/ml) vs. the scale in Fig. 2A-D (0-700 pg/ml).

SAL challenge had no effect, whether preceded by a two-week NIC infusion or not (Fig. 2A,B). Continuous NIC infusion desensitized the response to single-dose NIC (0.3 mg/kg), reducing the ACTH response in females by more than half and eliminating it in males (Fig. 2C,D), a highly significant difference (main effect of sex: $F(1, 148) = 25.1$; $p < 0.0001$). MEC pretreatment enabled a small response to SAL in females but not in males (main effect of sex: $F(1, 136) = 16.5$; $p < 0.0001$) (Fig. 2A,E), and continuous NIC infusion prior to MEC pretreatment enabled a small response to SAL in males as well (Fig. 2F). Single-dose NIC administration following MEC pretreatment produced an ACTH response in both sexes that was only slightly stronger than the response to SAL following MEC (Fig. 2F,G). Continuous NIC infusion prior to MEC pretreatment attenuated the ACTH response to single-dose NIC administration in males but enhanced it in females (Fig. 2G,H). Multiple comparisons at each time point between sexes, at each time point compared to baseline, and at each time point compared to SAL control are indicated in Fig. 2.

CORT—Fig. 3 shows plasma CORT concentrations (ng/ml) following SAL (Fig. 3A) and increasing doses of NIC (Fig. 3B-D). As mentioned above, all groups were administered SAL at -20 min, followed by SAL (1 ml/kg) or NIC (0.1, 0.3, or 0.5 mg/kg) at 0 min. Baseline CORT values were significantly higher in females than in males in all groups. NIC stimulated CORT in both sexes, with little effect of dose other than a more prolonged CORT response following the highest NIC dose. Females showed greater CORT responses than did males, as indicated by a highly significant sex $\times$ drug interaction ($F(3,145) = 8.9$; $p < 0.0001$), as well as by highly significant main effects of both drug ($F(3,145) = 28.1$; $p < 0.0001$) and sex ($F(1, 145) = 236.7$; $p < 0.0001$). Multiple comparisons at each time point between sexes, at each time point compared to baseline, and at each time point compared to SAL control are indicated in Fig. 3.

Fig. 4 shows plasma CORT concentrations (ng/ml) following single-doses of SAL and NIC (0.3 mg/kg) administered after continuous NIC infusion and/or MEC pretreatment. As mentioned above, NIC (0.3 mg/kg) was used as the single-dose challenge, based on the sex difference in the ACTH response it produced. For the experiments in Fig. 4A-D, SAL was given at -20 min followed by SAL (1 ml/kg) or NIC (0.3 mg/kg) at 0 min. For the experiments in Fig. 4E-H, MEC (5 mg/kg) was given at -60 min followed by SAL (1 ml/kg) or NIC (0.3 mg/kg) at 0 min. For the experiments in Fig. 4B, D, F, and H, NIC was infused for two weeks via osmotic mini-pumps prior to single-dose SAL or NIC challenge. Note the expanded ordinate scale in Fig. 2E-H (0-1000 ng/ml) vs. the scale in Fig. 2A-D (0-1200 ng/ml).

SAL challenge, whether preceded by a two-week NIC infusion or not, had a small effect on CORT which was significantly greater in females (Fig. 4A,B). Continuous NIC infusion desensitized the CORT response to single-dose NIC (0.3 mg/kg) in males, as it did with ACTH, but it prolonged the response in females (Fig. 4C,D), a highly significant difference (sex $\times$ time interaction ($F(4, 596) = 4.22$; $p = 0.002$). MEC pretreatment enabled a significant CORT response to SAL in females but not in males (Fig. 4A,E), and continuous NIC infusion prior to MEC pretreatment enabled a small response to SAL in males as well (Fig. 4E,F). Single-dose NIC administration following MEC pretreatment produced a CORT response in both sexes, but this response was slightly stronger than the response to SAL following MEC only in the males (Fig. 4E,G). Continuous NIC infusion prior to MEC pretreatment attenuated the CORT response to single-dose NIC administration in males and

enhanced it in somewhat in the females (Fig.4G,H). Multiple comparisons at each time point between sexes, at each time point compared to baseline, and at each time point compared to SAL control are indicated in Fig. 4. Overall, other than baseline CORT being significantly higher in the females, the CORT responses to NIC and MEC treatments were similar to the ACTH responses.

IV. DISCUSSION

To our knowledge, this is the first study to explore the effects of single-dose NIC, continuous NIC infusion, and MEC induction of NIC withdrawal on sexually diergic HPA responses in rats. The results highlight sex differences in the effects of NIC, NIC habituation, and NIC withdrawal on HPA axis activity and stress responsiveness. They have implications for future animal studies of the biological processes underlying NIC addiction and withdrawal, and how these processes relate specifically to men versus women.

The sexually diergic ACTH responses to increasing doses of single-dose NIC support and extend our previous findings [57]. HPA axis responses to cholinergic stimulation by NIC are sexually diergic: Females have greater ACTH and CORT responses than males, as indicated by absolute plasma ACTH and CORT values. The present study determined ACTH and CORT responses to single-dose NIC by extending blood sampling to +60 min, as opposed to our previous study, in which blood sampling was terminated at +30 min [57]. As well, the present study used the average of two baseline blood samples at -25 and -15 min to yield a more reliable baseline value of -20 min; in contrast, our previous study only determined a single baseline value at -10 min.

Following continuous NIC, ACTH responses to single-dose NIC were blunted to a significantly greater degree in males than in females, suggesting that males may show greater habituation to NIC at or above the level of the pituitary gland. ACTH responses to NIC following continuous NIC were increased only in females, and CORT responses were increased to a greater extent in females than in males. CORT responses were increased in females following MEC/SAL, contrary to our previous findings, in which neither ACTH nor CORT responses to MEC/SAL were significantly altered [58]. Because the magnitude of the CORT changes appear to be similar in both studies, the difference in statistical significance is likely due to the considerably larger sample sizes in the present study.

The results of this study also corroborate previous reports on the time- and dose-dependent effects of single-dose and continuous NIC on ACTH and CORT secretion [20,24]. Male rats exposed to NIC (0.3 mg/kg) self-administration showed higher ACTH and CORT concentrations on Day 1 of exposure compared to Day 3 of exposure [20]. Chronic administration of NIC (0.1-0.5 mg/kg) to male rats by repeated injections reduced ACTH and CORT levels, and single-dose NIC (0.1-0.5 mg/kg) following chronic NIC administration no longer increased these hormones [10,15,40,67]. Continuous NIC (6 mg/kg/day) administered by mini-pump for 14 days increased ACTH and CORT responses only in female rats [24]. Taken together, these and our results demonstrate that the male HPA axis may be more sensitive to the habituating effects of continuous NIC than the female HPA axis [24].

Further, expression of neuronal nitric oxide synthase (nNOS), the enzyme responsible for catalyzing the conversion of L-arginine to nitric oxide (NO), appears to increase in the female rat hippocampus in response to chronic NIC (0.4 mg/kg subcutaneously for 15 days) [34]. Despite the fact that Keser et al. [34] injected 1/10 of the amount of NIC administered by osmotic pump in our study, based on their results and our current results we can hypothesize the following mechanism: The hippocampus can suppress HPA axis activity

[56,59]. Hippocampal nNOS expression appears to increase during times of stress, and it is possible that increased nNOS expression correlates with inhibition of hippocampal circuitry that suppresses HPA axis activity[34]. Chronic NIC administration increases hippocampal nNOS and NO only in female rats, resulting in greater hippocampal disinhibition, and therefore greater ACTH and CORT responses in females than in males. A complementary mechanism may involve the stimulatory effect of NO on other brain areas protected by the BBB; e.g., the paraventricular nucleus, wherein CRH and AVP are produced [61]. Stimulation of CRH and AVP by increased NO levels also would enhance the ACTH and CORT response to continuous NIC administration in female rats.

With reference to sexually diergic behavioral effects, NIC produces a bimodal response in social interaction tests, anxiolytic effects being produced by low doses (0.01-0.1 mg/kg, i.p.) and anxiogenic effects being produced by higher doses (0.5-1.0 mg/kg, i.p.) [26,40]. Caldarone *et al.* studied behavioral responses to NIC by administering continuous NIC in drinking water (0, 50, 100, and 200 µg/ml) for 4 weeks to male and female mice [16]. The mice then were placed in an elevated plus maze (EPM) for behavioral testing. Continuous NIC produced an anxiogenic response in females but failed to produce an anxiogenic response in males [16]. Because the increases in stress-responsive hormones suggested an anxiogenic physiological profile, these behavioral results appear consistent with the physiological findings of the present study, in which enhanced ACTH and CORT responses to NIC (0.3 mg/kg) following continuous NIC were observed to a greater extent in the females.

Withdrawal precipitated by MEC in rats treated with continuous NIC causes similar behavioral symptoms to those occurring in withdrawal from opiates [13,30,31,38]. In a study of men and women designated as smokers or non-smokers, naltrexone, an opioid receptor antagonist, or placebo was administered to induce NIC withdrawal, and HPA axis responses were determined [2]. Naltrexone increased ACTH and cortisol responses to a greater extent in non-smokers than in smokers [2]. Cortisol responses progressively increased following naltrexone administration [2], similar to our MEC paradigm, wherein CORT responses were sustained over time after MEC/SAL following continuous NIC. Another human study using a naltrexone model of withdrawal showed that ACTH and cortisol responses in male and female smokers were increased only in the women [63]. The similarities in HPA hormone results of these two human studies and our present study are interesting, but the use of different withdrawal agents (naltrexone vs. MEC) makes comparisons difficult.

It also is possible that the hormone responses in our study were pharmacologically driven by MEC itself at the dose we used (5 mg/kg). A NIC abstinence syndrome characterized by behavioral effects consistent with NIC withdrawal occurs at much lower doses (e.g., 1 mg/kg) [30,38,69], and the selectivity of MEC for nicotinic receptors may be compromised at doses higher than 1 mg/kg [40]. Consequently, our MEC dose (5 mg/kg) may have produced direct pharmacological as well as NIC withdrawal effects on the HPA axis.

Given the high lipophilicity of NIC, its brain distribution would be expected to be comparable between the sexes [36]. Several other methodological factors, however, may have contributed to the sex differences in hormone responses. First, pharmacokinetic differences may have influenced our results: Female rats have a larger volume of distribution of NIC than do male rats, and males have a shorter NIC half-life [36]. Second, sexually diergic NIC metabolism [40] and sexual dimorphism of the BBB [48,65] may have influenced the entry of NIC into the brain; to our knowledge there are no published data that address this issue. Third, structures lying outside the BBB that are susceptible to cholinergic activation, including the pituitary, ovaries, and testes [25,53,70,71], may have differentially

influenced the release of hormones in males and females. Finally, continuous NIC exposure results in neuroadaptation, the development of tolerance to NIC associated with an increased number of brain nicotinic cholinergic receptors [8], which occurs to a greater extent in males than in females [16,35,45]. Smoking cessation is more difficult for women than for men, which might be due to faster metabolism of NIC in women, resulting in a greater incidence of NIC addiction in women than in men [8,9].

HPA axis activation has been shown to involve norepinephrine (NE) release into the PVN, subsequent to activation of brainstem catecholaminergic regions by NIC [39,41,57]. NIC also activates adenylatecyclase and increases cytosolic calcium in AVP neurons in the SON in male rats, although less potently than NE [57,68]. In support of the SON/AVP mechanism, bilateral injection of MEC into the PVN failed to prevent ACTH responses to systemic NIC [39,42,57]. Nicotinic receptor subunits have been identified on CRH-containing neurons in the median eminence, so it is possible that systemic NIC had a direct effect on the median eminence, resulting in CRH stimulation [27,47]. Of importance, any direct effects of NIC on the sexually diergic hormone responses in the present study must remain speculative, because the studies discussed above used only male animals [59].

Nicotinic receptors in extra-hypothalamic brain areas also may have influenced the sexually diergic modulation of HPA axis activity in our study. Nicotinic receptors are found in the hippocampus and amygdala, pertinent areas for HPA axis regulation [28,32,37,57,72]. A single IV injection of NIC (0.1 mg/kg) induced Fos-like immunoreactivity in numerous extrahypothalamic brain regions, as well as in the PVN and SON [57,66], but again only male rats were studied. The potential sexual diergism of c-fos induction and Fos expression following NIC administration remains to be studied, as do sex differences in nicotinic receptor distribution in brain areas relevant to HPA axis regulation.

The hormone results for our control groups that were implanted with osmotic mini-pumps filled with SAL show that baseline ACTH and CORT levels were not increased in these groups (unpublished data), suggesting that the stress of surgery and pump implantation did not influence our results. We used the Model 2002 Alzet osmotic mini-pump to deliver NIC (4.5 mg/kg/day) i.p. to Sprague-Dawley rats weighing 200-225 g. We selected this pump and NIC dosage to approximate NIC concentrations in smokers consuming approximately one-half to one pack of cigarettes per day and to be consistent with our previous single-dose NIC studies using JVC young adult male and female rats. We did not measure plasma NIC concentrations, because the stability of NIC in the mini-pumps and the steady-state plasma NIC concentrations following implantation of mini-pumps containing NIC have been reliable and reproducible [11,12,31]. As well, stable plasma NIC levels (approximately 25 ng/ml) have been demonstrated in rats receiving continuous NIC (1 mg/kg/day) by osmotic mini-pump, corresponding to plasma NIC levels in habitual smokers [11,40]. Future studies with our NIC-administration paradigm in which plasma NIC concentrations are quantified will be important to define the pharmacodynamic and pharmacokinetic contributions to NIC-induced HPA responses in males and females.

In conclusion, our study demonstrates that HPA axis responses to stimulation by single-dose and continuous NIC, as well as MEC-induced withdrawal, are sexually diergic: Female ACTH and CORT responses are greater than male responses. Following continuous NIC, HPA responses to single-dose NIC challenge were significantly lower in magnitude and duration, and the sex difference remained. As well, female CORT responses were significantly greater than male responses to MEC-precipitated withdrawal. Our findings indicate that sexually diergic HPA responses to single-dose NIC administration are reduced and transient following continuous NIC, but are enhanced and sustained following NIC withdrawal. This study highlights the importance of sex differences in the effect of NIC on

HPA axis activity and stress responsiveness, which may have implications as an animal model for investigating specific treatments for NIC addiction in men and women.

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Figure 1. ACTH dose-response to NIC or SAL in male and female rats

Baseline blood samples were collected at -25 and -15 min to yield an average baseline (-20 min). SAL (1 ml/kg) was given IP at -20 min, and SAL or NIC (0.1, 0.3, or 0.5 mg/kg) was given IP at 0 min. Additional blood samples were collected at +10, +20, +40, and +60 min. Each bar represents the mean $\pm$ SEM of 15 to 24 rats. N's by sex for each group are SAL (M = 24, F = 23); NIC 0.1 (M = 15, F = 15); NIC 0.3 (M = 18, F = 16); NIC 0.5 (M = 18, F = 17). Tukey-Kramer multiple comparisons: *a*, sex difference at indicated dose and time ($p < 0.05$); *b*, indicated time vs. baseline ($p < 0.05$); *c*, indicated time vs. SAL control at same time ($p < 0.05$). * = letter applies to both sexes at the indicated time.

Figure 2. ACTH responses to SAL, single-dose NIC, and withdrawal by single-dose MEC, alone and following continuous NIC in male and female rats

Baseline blood samples were collected at -25 and -15 min to yield an average baseline (-20 min), or at -65 and -15 min to yield an average baseline (-40 min). SAL (1 ml/kg) was given IP at -20 min (A-D); MEC (5 mg/kg) was given IP at -60 min (E-H); and SAL or NIC (0.3 mg/kg) was given IP at 0 min (A-H). NIC was infused continuously for two weeks prior to experimentation (B, D, F, H). Additional blood samples were collected at $+10$, $+20$, $+40$, $+60$ min. Each bar represents the mean $\pm$ SEM of 15 to 24 rats. N's by sex for each group are SAL (M = 24, F = 23); SAL following NIC (M = 17, F = 17); NIC 0.3 (M = 18, F = 16); NIC 0.3 following continuous NIC (M = 17, F = 17); MEC/SAL (M = 20, F = 17); MEC/SAL following continuous NIC (M = 15, F = 16); MEC/NIC 0.3 (M = 17, F = 17); MEC/NIC 0.3 following continuous NIC (M = 18, F = 17). Tukey-Kramer multiple comparisons: *a*, sex difference at indicated dose and time ($p < 0.05$); *b*, indicated time vs. baseline ($p < 0.05$); *c*, indicated time vs. SAL control at same time ($p < 0.05$); NIC = continuous NIC (4.5 mg/kg/day) infusion by Alzet® osmotic mini-pump. * = letter applies to both sexes at the indicated time.

Figure 3. CORT dose-response to NIC or SAL in male and female rats
See Fig. 1 legend for explanation.

Figure 4. CORT responses to SAL, single-dose NIC, and withdrawal by single-dose MEC, alone and following continuous NIC in male and female rats
See Fig. 2 legend for explanation.