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Sexually Diergic, Dose-Dependent Hypothalamic-Pituitary-Adrenal Axis Responses to Nicotine in a Dynamic *In Vitro* Perfusion System

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Abstract

Introduction—The hypothalamic-pituitary-adrenal cortical (HPA) axis modulates physiological responses to stress. We previously reported sexually diergic, dose-dependent HPA responses *in vivo* following nicotine administration: Male rats had greater arginine vasopressin (AVP) responses than females, and female rats had greater adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) responses than males. The goal of the present study was to further investigate sexually diergic, dose-dependent HPA responses following nicotine addition to an *in vitro* model of the HPA axis, so that hormone output could be determined at each level of the axis.

Methods—Hypothalami, pituitaries, and adrenal glands were harvested from male and female rats. One-half hypothalamus, one-half pituitary, and one adrenal gland were placed individually into three jacketed tissue baths connected by tubing and perfused in series with physiological medium. Sampling ports between tissue baths were used to collect buffer before and after addition of various doses of nicotine, for measurement of AVP and corticotropin-releasing hormone (CRH) from the hypothalamus bath, ACTH from the pituitary bath, and CORT from the adrenal bath. Hormones were measured by highly specific immunoassays.

Results—Stable temperatures, flow rates, pH, and hormone baselines were achieved in the *in vitro* system. Consistent with our *in vivo* and earlier *in vitro* studies, nicotine added to the hypothalamus tissue bath significantly increased HPA responses in a sex- and dose-dependent manner: Males had greater AVP responses than did females, and females had greater CRH responses than did males. Sexually diergic ACTH and CORT responses were less apparent and were higher in females.

Discussion—Our *in vitro* system accurately models *in vivo* HPA responses to nicotine in both sexes and thus represents a reliable method for investigating the effects of nicotine on components of the HPA axis. These studies may be pertinent to understanding the biological differences to NIC between men and women smokers.

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Keywords

ACTH; AVP; Cholinergic; CORT; CRH; Hormones; HPA Axis; Methods; Nicotine; Rat

1. Introduction

Cigarette smoking is a major worldwide health concern for both men and women (Benowitz, 2008; Rose, 2008). Nicotine (NIC) administration via various routes increases stress hormone concentrations in both humans (al'Absi, 2006; Mendelson et al., 2008; Mendelson et al., 2005; Pomerleau et al., 2004; Rohleder & Kirschbaum, 2006) and rodents (Cam & Bassett, 1983; Cam et al., 1979; Fu et al., 1997; Matta et al., 1987; Yu et al., 2008). Biological sex appears to be an important factor underlying stress hormone sensitivity following NIC (Faraday et al., 2005). Therefore, basic studies of sex differences in stress hormone responses to NIC are relevant to the understanding of biological differences between men and women smokers.

The hypothalamic-pituitary-adrenal (HPA) axis is a three-gland component of the endocrine system that modulates biological responses to acute and chronic stress (Bugajski et al., 1999). Immediately following stress, HPA axis activity increases, initiated by the release of corticotropin-releasing hormone (CRH) from neurons of the paraventricular nuclei of the hypothalamus. CRH, in turn, stimulates adrenocorticotrophic hormone (ACTH) release from the anterior pituitary, and ACTH stimulates corticosterone (CORT) release from the adrenal cortex. Arginine vasopressin (AVP), released from neurons of the paraventricular and supraoptic nuclei of the hypothalamus, acts as a potent secretagogue of ACTH release, potentiating the CRH-driven stress response (Aguilera et al., 2008; Antoni, 1993; Engelmann et al., 2004; Surget & Belzung, 2008; Volpi et al., 2004).

Several neurotransmitters, including acetylcholine (ACh), modulate HPA axis activity (Rhodes et al., 2001b; Rhodes & Rubin, 1999; Whitnall, 1993) and are released in response to NIC (Albuquerque et al., 2000; Benowitz, 2008; Paterson, 2000). NIC, the main psychoactive component of cigarettes, has been shown to activate the HPA axis in a dose-dependent manner (Mendelson et al., 2005; Porcu et al., 2003; Rhodes et al., 2004; Rhodes et al., 2001a). Reports of sex differences in HPA axis responses to NIC are relatively few (e.g., (Faraday et al., 2005; Grota et al., 1997; Moidel et al., 2006; Pogun & Yazarbas, 2009; Rhodes et al., 2004). Our previous *in vivo* studies indicate that NIC activates the HPA axis in a sexually diergic manner, males having significantly greater plasma AVP responses than females, and females having significantly greater plasma ACTH and CORT responses than males (Rhodes et al., 2001a).

We previously reported that, in a dynamic perfusion system of HPA tissues, NIC also activates the HPA axis in a sexually diergic manner (Moidel et al., 2006). However, only one NIC dose was used in the previous study and AVP was not measured. The purposes of the present study were (1) to further validate this *in vitro* model with improved system components and physiological parameters; (2) to determine sexually diergic CRH, AVP, ACTH, and CORT responses to cholinergic stimulation by several doses of NIC; and (3) to compare the results with previously determined *in vivo* and *in vitro* hormone responses to NIC stimulation.

2. Methods

2.1. Animals

All animal procedures were approved by the Saint Vincent College Animal Care and Use Committee and were conducted in accordance with National Institutes of Health guidelines for proper animal care. Eight-week-old male and female Sprague-Dawley rats weighing 200–225

g were obtained from Taconic Farms, Inc. (Germantown, NY, USA). All animals were housed doubly in a well-ventilated, temperature- and humidity-controlled environment (22–25°C, 50–75% humidity) and were maintained on a 12-h light-dark cycle (lights on at 0800 h) with food and water available *ad libitum*.

2.2. Tissue Isolation

Rats were euthanized by isoflurane (Baxter, Deerfield, IL, USA) inhalation prior to decapitation, methods consistent with the recommendations of the Panel of Euthanasia of the American Veterinary Medical Association. Immediately following decapitation, the hypothalamus, pituitary gland, and adrenal glands were collected. The hypothalamus was isolated by the block method (Hatton et al., 1980). The pituitary was collected from the sella turcica after severing the optic nerves and infundibulum and removing the brain from the skull. The hypothalamus and pituitary were bisected prior to weighing. Adrenal glands were removed by ventral approach and were cleared of adipose tissue. Tissue isolation time was approximately five minutes for each animal.

Each hypothalamic and pituitary tissue-half and each adrenal gland was weighed and placed individually in beakers containing modified Bradbury tissue culture medium (pH 7.4) at 37°C (Bradbury et al., 1974; Garrido et al., 1999). The culture medium contained (in mM) 126 NaCl, 6 KCl, 0.88 MgSO₄, 1 Na₂HPO₄, 22 NaHCO₃, 1.45 CaCl₂, 11 glucose, and 0.05 ascorbic acid (all reagents from Sigma, St. Louis, MO, USA). The tissues were immediately enclosed in 100 mesh, 20 mm (3/4") circular, stainless-steel screens (Small Parts, Inc., Miami Lakes, FL, USA) that were folded in half and held together with 9 mm stainless-steel wound clips (World Precision Instruments, Sarasota, FL, USA). These were transferred to 10-ml jacketed tissue baths (World Precision Instruments, Sarasota, FL, USA) comprising the *in vitro* perfusion system (Fig. 1). The hypothalamus baths were connected to the source buffer flask via Tygon flexible plastic tubing (3.2 mm [1/8"] I.D.; 6.4 mm [1/4"] O.D.; 1.6mm [1/16"] wall thickness). The jacketed tissue baths and a terminal siphon regulator were connected to each other by Tygon flexible plastic tubing (3.2 mm [1/8"] I.D.; 4.8 mm [3/16"] O.D.; 0.8 mm [1/32"] wall thickness) (Cole-Palmer, Vernon Hills, IL, USA) with Luer connectors (Cole-Palmer, Vernon Hills, IL, USA) and 3-way stopcocks (World Precision Instruments, Sarasota, FL, USA), to enable the connection of sampling ports composed of 3.2 mm Tygon tubing plus Luer connectors (Cole-Palmer, Vernon Hills, IL, USA). The interior surface of each 10-ml jacketed tissue bath was coated with silicone lubricant (Dow Corning Corp., Midland, MI, USA) to prevent ACTH from binding to the glass. Each jacketed tissue bath was oxygenated via Tygon tubing (3.2 mm [1/8"] I.D.; 6.4 mm [1/4"] O.D.; [1/16"] wall thickness) connected to a polycarbonate multi-port manifold (Cole-Palmer, Vernon Hills, IL, USA). The flow rate between baths was regulated by the speed of the perfusion pump (Minipuls 3, Gilson), by changing the height of the tissue baths in relation to one another, and by the terminal siphon regulator which was connected to the terminal end of the adrenal jacketed tissue bath via Tygon tubing. The terminal siphon regulator ensured a steady flow rate out of the adrenal jacketed tissue bath, and thus through the entire *in vitro* system. A Bechman Centerline II circulating water bath was used to circulate water (38 °C) through the jackets surrounding the tissue baths. The tissues were equilibrated by perfusion of 37° C culture medium for at least 30 min before experiments were initiated.

2.3. In Vitro System

Modified Bradbury culture medium warmed (49–60 °C; depending on ambient room temperature, flow rate through the system, and tissue bath temperatures) and buffered (pH 7.3–7.5) at the 1L source flask was perfused through the system by a perfusion pump (Gilson Minipuls 3, Middleton WI, USA) (Fig. 1). A 1L reserve flask containing warmed culture medium was used to replenish the medium of the source flask as necessary during the

experiments. Each jacketed tissue bath was gassed with 95% O₂, 5% CO₂ and had a total medium volume of approximately 6ml. Oxygenation flow levels were controlled in each jacketed tissue bath via Keck Ramp Clamp Tubing Clamps (Cole-Parmer, Vernon Hills, IL, USA). Including volume loss from the system during sampling, flow rates of the medium through the system during the experiments ranged between 1.1–1.9 ml/min. Temperatures ranged between 36.1°–38.4°C in hypothalamus baths, 37.1°–38.5°C in pituitary baths, and 36.2°–38.9°C in adrenal baths. At the end of each experiment, the tissues were exposed to 60 mM KCl for 10 min to test tissue responsiveness and viability to membrane depolarization (Gao et al., 2000). Temperature and pH of buffer in the source flask, the reserve flask, and each jacketed tissue bath were continuously measured throughout each experiment with alcohol thermometers (Allegiance, McGraw Park, IL, USA), individual digital thermometers (VWR, West Chester, PA, USA) and pH electrodes and meters (Radiometer PHM92, Cedex, France). Due to heating, the pH of the Modified Bradbury buffer gradually rose throughout experimentation. The pH of the buffer was thus regulated via drop-wise additions of 1 N HCl.

2.4. Sampling for Stability of Baseline Hormone Concentrations

At the beginning of each experiment, baseline culture medium samples were taken from each of the sampling ports at 0, 5, 10, 15, and 20 min following the 30 min tissue equilibration for determination of CRH, AVP, ACTH, and CORT concentrations. Additional baseline culture medium samples were taken prior to each NIC administration. Following NIC stimulations at 70, 110 and 150 min, hormone concentrations returned to baseline by 100, 140, and 180 min, consistent with our earlier findings (Moidel et al., 2006). The duration of each experiment was 200 min (Fig 2).

2.5. NIC Addition and Sampling

Drug solutions were freshly prepared immediately prior to each experiment. For the 50 ng/ml dose, NIC (nicotine hydrogen tartrate salt; Sigma, St. Louis, MO, USA) was dissolved in distilled water at a concentration of 1.5 mg in 1L (3 μ M). Three 0.2 ml aliquots (each 0.2 nmol free base NIC), separated by 1 min each, were added to each hypothalamus bath. The 3 NIC aliquots (0.6 nmol total free base NIC) yielded a final bath concentration of 50 ng/ml, which approximates the plasma NIC concentration after smoking one cigarette (Cam et al., 1979). Because of the high lipid solubility of NIC, plasma NIC concentrations reflect brain NIC concentrations (Chang et al., 2005).

For the 150 ng/ml dose, NIC was dissolved in distilled water at a concentration of 4.5 mg in 1 L (9 μ M). Three 0.2 ml aliquots (each 0.6 nmol free base NIC), separated by 1 min each, were added to each hypothalamus bath. In this case, the 3 NIC aliquots (1.8 nmol free base NIC) yielded a final bath concentration of 150 ng/ml. For the 200 ng/ml dose, NIC was dissolved in distilled water at a concentration of 6 mg in 1 L (12 μ M). Three NIC aliquots (each 0.8 nmol free base NIC), separated by 1 min each, were added to each hypothalamus bath. In this case, the 3 NIC aliquots (2.4 nmol total free base NIC) yielded a total concentration of 200 ng/ml. Culture medium samples were collected prior to and 5, 10, and 20 min after NIC addition (n = 5–6 axes per group [3 tissue sets per sex]).

2.6. Hormone Assays

Culture medium samples were analyzed in singlet for CRH, AVP, ACTH, and CORT. CRH was analyzed by enzyme immunoassay (EIA) (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA). Inter- and intra-assay coefficients of variation were 5% and 14%, respectively. The minimum detectable CRH concentration was 5 pg/ml. AVP was determined by radioimmunoassay (RIA) (Alpco, Buhlmann Laboratories, Allschwil, Switzerland). Inter- and intra-assay coefficients of variation were 9.9% and 6.0%, respectively. The minimum detectable AVP concentration was 1.25 pg/ml. ACTH_{1–39} was determined by a highly specific

immunoradiometric assay (IRMA) (Diasorin, Stillwater, MN) (Raff & Findling, 1989). Inter- and intra-assay coefficients of variation were 5.74% and 10.7%, respectively. The minimum detectable ACTH concentration was 1.5 pg/ml. CORT was analyzed by RIA (MP Biomedicals, Orangeburg, NY, USA). Inter- and intra-assay coefficients of variation were 9.3% and 7.3%, respectively. The minimum detectable CORT concentration was 7.7 ng/ml.

2.7. Statistical analysis

Multiple *in vitro* experiments were performed under the same conditions to attain $n = 5-6$ per group (3 tissue sets per sex) (there was insufficient sample from one axis for female CRH determinations, therefore $N=6$ for male CRH results and $N=5$ for female CRH results. $N=6$ for male and female AVP, ACTH, and CORT results). To account for flow rate variation, CRH concentrations are reported as fmol/mg hypothalamus-half/min, AVP concentrations as amol/mg hypothalamus-half/min, ACTH concentrations as fmol/mg pituitary-half/min, and CORT concentrations as pmol/mg single adrenal gland/min. Data are reported as mean $\pm$ standard error of the mean (SEM), and were analyzed by multifactorial analyses of variance (ANOVA), with time as a repeated measure. All data sufficiently approximated a Gaussian distribution such that no transformations were required (log-transformation did not alter the statistical results). Fisher's LSD tests were used for *post-hoc* comparisons. Significance was considered as $P < 0.05$.

3. Results

3.1. Baseline Hormone Release

The *in vitro* perfusion system produced minimally fluctuating CRH, AVP, ACTH, and CORT baselines prior to NIC stimulation. Basal CRH, AVP, and ACTH concentrations before and during experiments (between NIC stimulations) were not significantly different. As well, hormone concentrations were consistent between replicate experiments. Baseline values corrected for perfusion-medium flow rate and tissue weight were similar to those reported previously (Moidel et al., 2006).

3.2. Tissue Viability

At the end of each experiment, infusion of highly concentrated potassium buffer significantly increased CRH secretion from both male and female hypothalami at 5 and 10 min (P 's < 0.05) (Fig 3). Potassium buffer increased AVP secretion from male and female hypothalami approximately 3-fold and 2-fold, respectively, at 10 min (Fig 3). ACTH and CORT concentrations also increased 10 min following potassium buffer infusion (data not shown), but these increases were not statistically significant. Thus, the hypothalamic, pituitary, and adrenal tissues appeared to remain viable throughout the experiments.

3.3. Hormone Responses to NIC

Following addition of NIC to the hypothalamus bath, CRH increased promptly, followed a short time later by AVP and ACTH. ACTH responses to NIC were less pronounced in both sexes, and there was no perceptible increase in CORT, possibly because the ACTH concentration reaching the adrenal bath was too low. CRH and AVP are presented both as absolute values (Fig. 4–5) and as % changes from baseline (Fig. 6) to highlight differences in CRH and AVP hormone profiles between the sexes. ACTH and CORT are presented as absolute values only (Fig. 7–8).

Fig. 4 presents the means and SEMs of CRH responses from the hypothalamus bath 5 minutes prior to and 5, 10, and 20 min following addition of vehicle or NIC to that bath. Vehicle control slightly and non-significantly increased CRH responses in both sexes. There were no

significant interactions among sex, NIC dose, or time. There was a significant main effect of time ($F = [3, 132] = 15.3$, $P < 0.0001$), reflected by both female and male CRH responses at 5 and 10 min compared to baseline values prior to NIC stimulation (P 's < 0.05). Female tissue showed a robust and significant response to the 50 ng/ml NIC dose (P 's < 0.05) that was not enhanced by higher NIC doses; in contrast, male CRH responses, which were about half the female CRH responses following the 50 and 150 ng/ml doses, increased to the level of the female responses following the 200 ng/ml dose.

Fig. 5 presents the means and SEMs of the AVP responses. Vehicle control slightly and non-significantly increased AVP responses in both sexes. AVP showed a significant NIC dose $\times$ time interaction ($F [9, 144] = 2.0$, $P < 0.04$), most likely owing to the nonlinearity of the combined male and female AVP responses at 20 min. Male tissue showed a robust and significant response to the 150 ng/ml dose that was not enhanced by the 200 ng/ml dose (P 's < 0.05); in contrast, female AVP responses, which were unaffected by the 150 ng/ml dose, increased nearly to the level of the male AVP responses following the 200 ng/ml dose.

Fig. 6 presents the mean % changes from baseline and SEMs for CRH (top graphs) and AVP (bottom graphs) at 5, 10, and 20 min following NIC addition to the hypothalamus bath. As with the absolute CRH changes, CRH % changes showed a significant main effect of time ($F [2, 88] = 8.34$, $P = 0.0006$). The CRH response was short-lived in both sexes, returning to baseline values by 20 min. AVP % changes from baseline also showed a significant main effect of time ($F [2, 96] = 3.48$, $P < 0.04$) and a significant dose $\times$ time interaction ($F [6, 96] = 2.4$, $P < 0.04$), reflected primarily in the male % changes at 10 and 20 min following the 150 and 200 NIC doses.

Fig. 7 presents the means and SEMs of the ACTH responses. Vehicle control slightly and non-significantly increased ACTH responses in both sexes. There were no significant interactions among sex, NIC dose, or time. ACTH showed a significant main effect of time ($F [3, 144] = 4.0$, $P < 0.009$): Following the 150 ng/ml dose, female ACTH responses were significantly higher at 5 and 10 min, and male ACTH responses were significantly higher at 10 and 20 min, compared to baseline values prior to NIC stimulation (P 's < 0.05). The main effects of both sex and dose were non-significant.

Fig. 8 presents the means and SEMs of the CORT responses. Baseline CORT values from female adrenals were higher than those from male tissues. There was no discernible effect of vehicle control on CORT. There also were no significant interactions among sex, NIC dose, or time. Following NIC (and ACTH) stimulation, there were significant main effects of time ($F [3, 144] = 4.3$, $P = 0.006$) and dose ($F [3, 47] = 5.21$, $P < 0.004$), reflecting varying decreases in male and female CORT responses with increasing NIC doses. There also was a significant main effect of sex ($F [1, 47] = 6.1$, $P < 0.02$): Female responses were significantly higher than male responses at most time points, based largely on the higher initial baseline values in females.

4. Discussion

In the present study, we replicated the ability of our *in vitro* perfusion system containing hypothalamic, pituitary, and adrenal tissues to model pharmacological responses of the HPA axis we have shown *in vivo*. Similar to our previous *in vitro* study, females had greater CRH and ACTH responses, although the ACTH responses were less perceptible (Moidel et al., 2006). Also in the present study, we were able to measure AVP and found that male responses were higher than female responses, in accord with our previous NIC *in vivo* studies. Also similar to these *in vivo* studies, female rats had greater, though less pronounced, ACTH and CORT responses than did males (Rhodes et al., 2001a). The present study determined hormone

responses to 50 ng/ml, 150 ng/ml and 200 ng/ml NIC, whereas our previous *in vitro* study only determined hormone responses to 50 ng/ml NIC.

Numerous *in vitro* studies have investigated hormone release following cholinergic and/or hormonal stimulation of isolated HPA axis tissues, as discussed extensively in our previous *in vitro* study (Moidel et al., 2006). However, no studies prior to or following our previous *in vitro* study have determined simultaneous, *multiple* hormone release from HPA tissues perfused in series. *In vitro* studies offer many advantages, including decreased animal use and elimination of difficult collection of portal blood for analysis of CRH and AVP (Aguilera et al., 2008; Wilhelmi et al., 2002). Our *in vitro* system also eliminates the influence of external factors that influence HPA axis activity, such as variable CNS input (Gray et al., 1989; Jacobson & Sapolsky, 1991; Madeira & Lieberman, 1995; Van de Kar & Blair, 1999); the stress of housing, handling, and experimentation (Belz et al., 2003; Brown & Grunberg, 1995; Brown & Grunberg, 1996; Gadek-Michalska & Bugajski, 2003); and peripheral sex hormones (Rhodes et al., 2002; Rhodes et al., 2004; Seale et al., 2004). It also eliminates negative feedback at the level of the hypothalamus and the pituitary from CORT, and can be adapted to add feedback in a controlled manner.

Several improvements to our previous *in vitro* model have made it possible to better emulate *in vivo* HPA axis physiology. The transition from Erlenmeyer flasks to jacketed tissue baths to hold the tissues allowed for greater temperature control of each bath within physiological ranges. For example, in our previous study, temperatures ranged from 32–34 °C in the adrenal flask, whereas the current system maintained temperatures from 36.2–38.9 °C for the duration of the experiments in the adrenal tissue baths. CORT concentrations in our previous study were higher in male tissues compared to female tissues, whereas in the current study CORT concentrations were higher in female tissues compared to male tissues, reflecting *in vivo* findings (Luine et al., 2007; Moidel et al., 2006; Rhodes et al., 2001a), perhaps due to better temperature control.

Another improvement is the reduction in perfusion flow rate to increase hormone concentrations during sampling from each bath. In our previous study, flow rate ranged from 4.5–6.0 ml/min, compared to 1.1–1.9 ml/min in the present study. This refinement was made in order to improve hormone measurements, particularly of AVP. Male AVP responses were higher than female responses, consistent with *in vivo* findings from our laboratory (Rhodes et al., 2004; Rhodes et al., 2001a). As well, in our previous *in vitro* study, culture medium was oxygenated at the source flask, which may have contributed to variable oxygen concentrations among the tissue baths. In the current study, each bath was oxygenated separately, enhancing tissue viability.

With the lower flow rates, AVP measurements were achievable, but CORT responses to NIC stimulation were less pronounced than in our earlier study. Because of the slower flow rate and the tendency of the pH of the perfusion medium to increase, the pH in the adrenal baths may not have been within the physiological range, affecting adrenal viability. Future studies will determine if a flow rate between those of the previous and present studies will allow measurable concentrations of all hormones as well as perceptible differences in CORT responses.

In the present study, bath volumes may not have approximated corresponding *in vivo* extracellular pools and therefore may have influenced hormone concentrations. Future studies will investigate differing bath sizes and volumes, to better approximate the expected extracellular pools of the specific tissues and therefore the drug and hormone concentrations surrounding them (e.g., a smaller pituitary bath to approximate the small volume of portal blood reaching it, and larger hypothalamic and adrenal baths to approximate the relatively larger volume of systemic blood reaching them).

NIC, as it perfused throughout the entire system, also may have differentially influenced male and female ACTH and CORT responses in the pituitary and adrenal tissues, respectively. Future studies will address the effects of NIC stimulation at various inter-bath points along the *in vitro* axis (NIC addition at the hypothalamic bath vs. addition at the pituitary bath vs. addition at the adrenal bath) to determine the direct effects of NIC on each tissue and perhaps better mimic delivery of NIC (and hormones) *in vivo*. Under non-stressful conditions, both CRH and AVP are released in a rhythmic, pulsatile fashion *in vivo*. The pulsations of these hormones increase in both amplitude and synchronization during stress (Chrousos, 1998; Chrousos, 2000; Chrousos & Gold, 1992). In our previous study, there were more perceptible differences in ACTH and CORT responses, perhaps because the flow of the buffer through the system was driven by gravity and the delivery of buffer to each bath was pulsatile in nature, as opposed to the continuous delivery of buffer in the present study. By dosing at inter-bath points along the *in vitro* axis, a concentrated, pulsatile delivery of drug can be achieved at each level of the axis, which may improve the ACTH and CORT responses measured after stimulation. Future studies using direct delivery of various doses of CRH and ACTH, to the pituitary and adrenal baths, respectively, also will address pituitary and adrenal sensitivity to these hormones.

In the current study, incremental dosing of NIC was chosen, because of the possibility of a large dose of NIC producing long-lasting effects that would mask subsequent responses to smaller doses of NIC. In order to truly explore the dose-responsiveness of NIC in our *in vitro* system, future studies will use a random dosing paradigm, allowing for sufficient time between doses for hormone concentrations to return to baseline. Future studies using random NIC dosing also may elucidate whether or not the tissues were primed or desensitized by incremental NIC dosing.

As stated previously, CRH responses were higher in female tissues compared to male tissues, and AVP responses were higher in male tissues compared to female tissues, similar to our *in vivo* studies (Rhodes et al., 2001a). As well, AVP % changes from baseline were more prolonged in males compared to females following the 150 ng/ml and 200 ng/ml NIC doses (Fig. 6). This suggests that following the higher doses of NIC, female and male hypothalami differ in both degree and timing of hormone responses to cholinergic challenge by NIC.

In an *in vivo* study of CRH/AVP mRNA co-expression in the parvocellular paraventricular nucleus (pcPVN) following self-administered NIC in male rats, CRH expressing neurons were markedly decreased, whereas AVP expressing neurons were increased in both the pcPVN and the magnocellular PVN (mcPVN). Further, the number of CRH neurons co-expressing AVP mRNA in the pcPVN increased fivefold (Yu et al., 2008). This shift in mRNA expression following self-administered NIC parallels the CRH vs. AVP responses observed in males in the present study.

Although Yu et al. (2008) studied only male rats, one can speculate that shifts from CRH to AVP mRNA expression and hormone release may have contributed to the sex differences in the timing and intensity of hormone responses that we observed. AVP responses appear to be sustained in male but not in female tissue, wherein responses appear to remain CRH-driven. Other studies also support the prolongation of AVP secretion in male rats (Aguilera et al., 2008; Volpi et al., 2004), and a shift to more than half of CRH neurons co-expressing AVP (Aguilera et al., 2007; Aguilera et al., 2008; Chowdrey et al., 1995; de Goeij et al., 1991; Ma et al., 1997; Volpi et al., 2004; Whitnall, 1993), but again, none of these studies specifically addressed sex differences, and they were conducted primarily in male rats.

Finally, current smokers tend to smoke more cigarettes when stressed, and following smoking cessation, women tend to relapse to smoking more quickly than men (al'Absi, 2006; Benowitz, 2008; Bjornson et al., 1995; Caldarone et al., 2008; Faraday et al., 2005; Pogun & Yazarbas,

2009; Pomerleau et al., 1991). Therefore, the potentially sexually diergic influence of nicotine on anxiety pathways could be explored in our *in vitro* system. For example, male and female amygdala tissue could be perfused with physiological buffer and stimulated with NIC, similar to the isolated HPA axis tissues in the present study.

In summary, our improved *in vitro* perfusion system supports the results of our previous *in vivo* and *in vitro* studies; NIC significantly increased HPA axis responses in a sex- and dose-dependent manner. Males had greater AVP responses than females, and females had greater CRH responses than males. Sexually diergic ACTH and CORT responses were less apparent. With the application of the aforementioned additional improvements to the *in vitro* system, sexually diergic ACTH and CORT responses should become more apparent. The ability of our *in vitro* system to parallel *in vivo* HPA axis responses to nicotinic challenge supports its potential as a reliable laboratory method in pharmacology and toxicology.

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Figure 1. In Vitro Perfusion System

The *in vitro* system models the hypothalamic-pituitary-adrenal (HPA) endocrine axis and consists of two sets of three jacketed tissue baths. The baths of each set contain one-half hypothalamus, one-half pituitary, and one adrenal gland connected in a series by tubing and accessories. Physiological buffer is perfused unidirectionally from a large Erlenmeyer source flask through the three baths to a waste beaker. Sampling ports are placed after each bath for sample collection before and after drug administration.

Figure 2. Timeline indicating additions to the in vitro system

Tissues were exposed to culture medium at 30 min, 50 ng/ml NIC at 70 min, 150 ng/ml NIC at 110 min, 200 ng/ml NIC at 150 min, and KCl at 190 min. The duration of each experiment was 200 min. Each tick mark represents 5 min.

Figure 3. In vitro CRH and AVP responses to high K⁺ buffer infusion

Tissues were exposed to 60 mM KCl at the end of each experiment to test tissue responsiveness and viability to membrane depolarization. Data are presented as 5 min prior and 5 and 10 min following infusion. b = difference from baseline (prior to K⁺ buffer addition) at indicated time point ($P < 0.05$). Each bar represents the mean $\pm$ SEM of 5–6 axes from 3 male and 3 female rats.

Figure 4. In Vitro CRH Dose-Responses to NIC in Male and Female Rat Tissues

CRH concentrations 5 min prior and 5 min, 10 min, and 20 min following NIC addition from 0–3 min (black bar). Each time point represents the mean $\pm$ SEM of 5–6 axes from 3 male and 3 female rats. a = sex difference at indicated time point, b = difference from baseline (prior to stimulation) at indicated time point, c = difference from control (buffer addition) at indicated time point, and * = difference in both sexes at indicated time point (P 's < 0.05).

Figure 5. In Vitro AVP Dose-Responses to NIC in Male and Female Rat Tissues

AVP concentrations 5 min prior and 5 min, 10 min, and 20 min following NIC addition from 0–3 min (black bar). Each time point represents the mean $\pm$ SEM of 6 axes from 3 male and 3 female rats. a = sex difference at indicated time point b = difference from baseline (prior to stimulation) at indicated time point, c = difference from control (buffer addition) at indicated time point, and * = difference in both sexes at indicated time point (P 's < 0.05).

Figure 6. Percent Changes from Baseline for In Vitro CRH and AVP Dose-Responses to NIC in Male and Female Tissues

% changes from baseline at 5, 10 and 20 min following buffer and the administration of NIC (0–3 min) in 0 ng/ml, 50 ng/ml, 150 ng/ml, and 200 ng/ml doses. Each time point represents the mean $\pm$ SEM of 5–6 axes from 3 male and 3 female rats. a = sex difference at indicated time point, b = difference from control at indicated time point, and c = difference from 5 min response at indicated time point.

Figure 7. In Vitro ACTH Dose-Responses to NIC in Male and Female Rat Tissues

ACTH concentrations 5 min prior and 5 min, 10 min, and 20 min following NIC addition from 0–3 min (black bar). Each time point represents the mean $\pm$ SEM of 6 axes from 3 male and 3 female rats. a = sex difference at indicated time point, b = difference from baseline (prior to stimulation) at indicated time point, c = difference from control (buffer addition) at indicated time point, and * = difference in both sexes at indicated time point (P 's < 0.05).

Figure 8. In Vitro CORT Dose-Responses to NIC in Male and Female Rat Tissues

CORT concentrations 5 min prior and 5 min, 10 min, and 20 min following NIC addition from 0–3 min (black bar). Each time point represents the mean $\pm$ SEM of 6 axes from 3 male and 3 female rats. a = sex difference at indicated time point, b = difference from baseline (prior to stimulation) at indicated time point, c = difference from control (buffer addition) at indicated time point, and * = difference in both sexes at indicated time point (P 's < 0.05).