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Nicotine self-administration diminishes stress-induced norepinephrine secretion but augments adrenergic-responsiveness in the hypothalamic paraventricular nucleus and enhances adrenocorticotrophic hormone and corticosterone release

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Abstract

Chronic nicotine self-administration augments the thalamo-pituitary-adrenal (HPA) responses to stress. Altered neuropeptide expression within corticotropin-releasing factor (CRF) neurons in the hypothalamic paraventricular nucleus (PVN) contributes to this enhanced HPA response to stress. Herein, we determined the role of norepinephrine, a primary regulator of CRF neurons, in the responses to footshock during nicotine self-administration. On day 12-15 of self-administration, microdialysis showed nicotine reduced PVN norepinephrine release by footshock (<50% of saline). Yet, the reduction in footshock-induced adrenocorticotrophic hormone (ACTH) and corticosterone secretion due to intra-PVN prazosin (α_1 adrenergic antagonist) was significantly greater in rats self-administering nicotine (2-fold) than saline. Additionally, PVN phenylephrine (α_1 agonist) stimulated ACTH and corticosterone release to a similar extent in unstressed rats self-administering nicotine or saline. Nicotine self-administration also decreased footshock-induced c-Fos expression in the nucleus of the solitary tract (NTS)-A2/C2 catecholaminergic neurons that project to the PVN. Therefore, footshock-induced NTS activation and PVN norepinephrine input are both attenuated by nicotine self-administration, yet PVN CRF neurons are more responsive to α_1 stimulation, but only during stress. This plasticity in noradrenergic regulation of PVN CRF neurons provides a new mechanism contributing to the HPA sensitization to stress by nicotine self-administration and smoking.

Keywords

nicotine; hypothalamic paraventricular nucleus; corticotrophin-releasing factor; norepinephrine; adrenocorticotrophic hormone; stress

Introduction

Corticotropin-releasing factor (CRF) neurons in the parvocellular division of the hypothalamic paraventricular nucleus (pcPVN) control the hypothalamo-pituitary-adrenal (HPA) axis, stimulating secretion of the critical hormones, adrenocorticotrophic hormone (ACTH) and, in turn, corticosterone (Herman *et al.* 2005). Norepinephrine (NE) is one of

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the primary pcPVN neurotransmitters that regulate ACTH secretion during stress (Pacak *et al.* 1995a). The pcPVN CRF neurons are innervated by noradrenergic neurons located in the nucleus of the solitary tract (NTS) (Cunningham and Sawchenko 1988). Adrenergic receptors, especially the α_1 subtype, are highly expressed in the pcPVN (Cummings and Seybold 1988), and specifically on CRF neurons (Day *et al.* 1999, Sands and Morilak 1999). Pharmacological studies have shown that the depletion of PVN NE or administration of prazosin, an α_1 adrenergic antagonist, inhibited plasma corticosterone responses to restraint stress (Gibson *et al.* 1986). Furthermore, microinjection of NE into the PVN increased corticosterone levels and induced c-Fos in the pcPVN (Cole and Sawchenko 2002). Therefore, NE inputs activate the pcPVN CRF neurons and mediate part of the HPA stress response through α_1 adrenergic receptors.

Nicotine, the principal psychoactive component of tobacco, is a stressor that acutely elevates ACTH and corticosterone secretion, desensitizing after repeated injections and long-term self-administration (Matta *et al.* 1998, Chen *et al.* 2008). In contrast, chronic nicotine self-administration (Chen *et al.* 2008) and other stressors (Aguilera 1994) augment ACTH and corticosterone responses to novel stressors, e.g. mild footshock stress. We recently reported that chronic nicotine self-administration induced co-expression of AVP mRNA within CRF neurons and facilitated activation of these pcPVN CRF⁺/AVP⁺ neurons by footshock, thus potentiating CRF-dependent ACTH secretion by co-releasing AVP (Rivier and Vale 1983, Yu *et al.* 2008). Such phenotypic changes may contribute to the cross-sensitization of HPA responsiveness to novel stressors during nicotine self-administration. However, the mechanism(s) by which nicotine self-administration facilitates the PVN CRF⁺/AVP⁺ neuronal activation by footshock is still unknown.

The PVN NE release from the NTS noradrenergic neurons, which is acutely stimulated by nicotine acting through the NTS nicotinic cholinergic receptors, also depends on glutamate secretion and NMDA receptors in the NTS (Fu *et al.* 1997, Zhao *et al.* 2007). This PVN NE release stimulates hypophysiotropic CRF neurons, increasing ACTH secretion (Valentine *et al.* 1996, Matta *et al.* 1998). Additionally, chronic nicotine self-administration increased the PVN NE secretion during the maintenance phase of nicotine self-administration (day 9.2 $\pm$ 0.6) and thereafter (day 18.6 $\pm$ 0.8) (Fu *et al.* 2001), although NE responses declined during this time.

Based on the central role of NE in activation of the pcPVN CRF neurons by stressors and nicotine, we hypothesized that nicotine self-administration alter the noradrenergic control of CRF neurons, augmenting the HPA response to footshock. To evaluate this, we determined the effects of nicotine self-administration on: (1) footshock-induced PVN NE release; (2) footshock-induced ACTH and corticosterone release after blockade of the PVN α_1 adrenergic receptors by prazosin; (3) ACTH and corticosterone secretion induced by activation of the PVN α_1 adrenergic receptors by phenylephrine; and (4) induction of c-Fos within the NTS-A2 and -C2 catecholaminergic neurons by footshock. These experiments demonstrate that nicotine self-administration augments the PVN adrenergic responsiveness during stress, yet footshock-induced activation of the NTS catecholaminergic neurons and the PVN NE release are significantly reduced by nicotine self-administration. These observations provide the evidence of nicotine self-administration-induced plasticity in the noradrenergic inputs to the PVN and in the PVN responsiveness to NE during mild footshock stress.

Materials and Methods

Materials

(-)-Nicotine hydrogen tartrate (dose expressed as free base), xylazine, ketamine, EDTA, nomifensine maleate, NE hydrochloride, prazosin hydrochloride, and phenylephrine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Sodium dihydrogen phosphate monohydrate, 1-octanesulfonic acid, methanol, acetonitrile and phosphoric acid for the mobile phase were obtained from Fisher Scientific (Fair Lawn, NJ). Dual channel swivels, polyethylene buttons and metal springs for intravenous infusion were purchased from Instech Laboratories (Plymouth Meeting, PA). Cellulose fiber tubing and silica tubing for microdialysis probes were purchased from Spectrum (Laguna Hills, CA), and Polymicron Technologies (Phoenix, AZ), respectively. Operant chambers, circuit boards, interface modules and software for nicotine self-administration and grid floors for electrical footshock were purchased from Coulbourn Instruments (Allentown, PA).

Animals and surgeries

Adult male Sprague-Dawley rats (300-350 g, Harlan, Madison, WI) were given access *ad libitum* to standard rat chow and water. After acclimation to a reversed 12:12 hours light/dark cycle for 7 days, rats were anesthetized with xylazine-ketamine anesthesia (13:87 mg/kg, respectively, i.m.). Guide cannulae (20 gauge for microdialysis, or 23 gauge for microinjection) were stereotactically implanted bilaterally into the PVN (10° angle). Coordinates from bregma with a flat skull were: anteroposterior, -2.0 mm; dorsoventral, -7.5 mm; mediolateral, ± 0.3 mm (Paxinos and Watson 1986). After recovery (7 day), rats received jugular or both jugular and femoral catheters under xylazine/ketamine anesthesia and were then placed into operant chambers and freely fed by food placed directly on the cage floor. All procedures conformed to NIH guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center.

Nicotine self-administration

Nicotine self-administration procedure was conducted using our published protocol (Valentine *et al.* 1997). Briefly, Rats were given access to nicotine or saline self-administration 23 hours/day for 12-15 days, without prior training, priming or food deprivation. The final hour of the 12h lights-on cycle (i.e., 9:00-10:00 AM) was reserved for housekeeping tasks. The operant chamber contained 2 horizontal levers, and a green cue light above each lever was illuminated when nicotine was available. Lever presses were recorded and syringe pumps controlled by computers and interfaces, using L2T2 or Graphic State software. Pressing the active lever, randomly designated, elicited a computer-driven injection (i.v., 50 μ l/0.81 sec) of nicotine (0.03 mg/kg) or saline via jugular vein. Each injection was followed by a 7 sec period during which the green cue light was extinguished but nicotine was unavailable. Pressing the alternate (inactive) lever had no programmed consequence.

Mild footshock stress

Electric footshocks were delivered 4 hours after the beginning of a 23 hours self-administration session. A total of 5 shocks (0.6 mA, 0.5 sec per shock) were randomly delivered over 5 min through the grid floor.

Experimental protocols

In the first experiment, the effects of nicotine self-administration on footshock-induced NE release in the PVN were detected. On day 12 of nicotine (n = 10) or saline (n = 12) self-

administration, *in vivo* microdialysis was used to measure the PVN NE release. In the second experiment, the effects of bilateral PVN microinjections (100 nl/1 min) of the α_1 adrenergic antagonist, prazosin (0.5 nmol/side), or vehicle (artificial CSF), delivered 10 min prior to footshock, on plasma ACTH and corticosterone responses to footshock were determined. In the third experiment, the effects of phenylephrine microinjection (α_1 adrenergic agonist; 10, or 40 nmol/side of PVN) or vehicle on ACTH and corticosterone secretion were evaluated. In the fourth experiment, the effects of ACTH₁₋₂₄ (15 or 300 nmol/kg, i.v.) or vehicle (0.5% BSA-containing saline) on corticosterone secretion were evaluated. The dose of prazosin, phenylephrine, and ACTH₁₋₂₄ are based on previous reports (Hwang *et al.* 1998, Ma and Morilak 2005, Ait-Chaoui *et al.* 1995). In the second through the fourth experiments, rats self-administering nicotine (n = 16) or saline (n = 21) were used to determine the effects of drug or vehicle on self-administration day 12 and 15; the order of drug vs. vehicle treatment alternated between animals. To diminish the stress of microinjection, bilateral cannulae (30 gauge) were inserted 1-2 hours before the onset of self-administration session through PVN guide cannula. Microinjections were delivered, using a microsyringe infusion pump, 4 hours after the self-administration session was initiated. Animals remained in their home operant chambers and were not handled during microinjection or i.v. injection. Two baseline blood samples (0.2 ml at -15 and 0 min) were withdrawn from the femoral vein prior to footshock, a microinjection of phenylephrine or i.v. injection of ACTH₁₋₂₄, and then 3 consecutive samples (15, 35, 55 min) were collected. An equivalent volume of saline, containing 100U heparin for maintenance of catheter patency, was injected immediately thereafter. In experiments 1-4, animals were killed with a lethal dose of isoflurane inhalation anesthesia. In the fifth experiment, immunocytochemical detection of c-Fos and dopamine β -hydroxylase (DBH) was used to assess the activation of the NTS noradrenergic and adrenergic neurons by footshock in rats self-administering nicotine (n = 8) or saline (n = 8) for 12 days. One hour after footshock or sham-shock, rats were killed, perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer, and then brains were removed, frozen and stored at -80°C.

***In vivo* microdialysis**

The microdialysis procedure was performed as described previously (Fu *et al.* 2001), using concentric microdialysis probes (1.5 mm dialysis membrane) constructed in our laboratory. Recovery efficiency for NE was 6.5 ± 0.6 % (n = 5). On the morning of self-administration day 12, 1-2 hours before the onset of the self-administration session, a probe was inserted through PVN guide cannula and perfused at 2 μ l/min with a solution of artificial CSF (140 mM NaCl, 2.5 mM KCl, 1.2 mM MgCl₂, 1.4 mM CaCl₂, 5 mM glucose, pH 7.2-7.4) containing 5 μ M nomifensine (NE reuptake blocker) (Schacht *et al.* 1982). Four hours after initiating the self-administration session, 3 consecutive baseline samples (10 min/sample; in 1 μ l 5% perchloric acid) were collected, footshocks were administered, and then 7 consecutive samples were obtained. All samples were stored at -80 °C. At the end of each experiment, probe positions were verified by histological examination. Only data obtained from animals with probes in the correct position were used for analysis of NE levels; 5 rats were excluded from a total of 27.

HPLC-electrochemical detection of NE

For HPLC, as previously reported (Zhao *et al.* 2007), the mobile phase (50 mM NaH₂PO₄·H₂O, 2.0 mM 1-octanesulfonic acid, 0.7 mM EDTA, and 10% methanol and 10% acetonitrile v/v in polished water, pH 6.0) was pumped through a C18 column (MDS 150 $\times$ 2 mm; ESA Inc., Chelmsford, MA) at 0.3 ml/min, using an ESA model 580 HPLC pump. Microdialysis samples were automatically injected by an autosampler (CMA microdialysis; North Chelmsford, MA). Electrochemical detection at 220 mV and 500 pA was performed with an ESA Coulochem II 500 electrochemical detector equipped with an ESA 5041 high-

sensitivity analytical cell. Serially diluted NE standards containing known quantities were included in each assay ($R^2 > 0.99$). The limit of detection for NE was 200 pg/injection.

Plasma corticosterone radioimmunoassay

Blood samples were collected into ice-cold tubes containing EDTA (20 mg/ml, 20 μ l), centrifuged at 830 g, 4°C, 10 min, and stored at -80°C. Corticosterone was measured on plasma samples, using the Immuchem™ Double Antibody Corticosterone 125 I RIA kit (MP Biomedicals, Orangeburg, NY), and minor modifications of published methods (Chen et al. 2008).

Immunocytochemical detection of dopamine β -hydroxylase (DBH) and c-Fos

Serial coronal brainstem sections (25 μ m) containing the the NTS-A2 (from bregma -13.68 to -14.3) and -C2 regions (bregma -13.24 to -13.68) (Paxinos and Watson 1986) were obtained by cryomicrotome (CM1850, Leica, Germany) and mounted on pre-cleaned slides. Briefly, dual labeling of neurons for DBH and c-Fos was obtained by sequentially incubating tissue sections with the following: 2% normal goat serum and normal horse serum in 0.01 M phosphate buffered saline with Tween-20 for 60 min; rabbit anti-c-Fos antibody (1:15,000; Calbiochem, San Diego, CA) and mouse anti-DBH antibody (1:1,000; Chemicon, Billerica, MA) overnight at 4°C; and biotinylated goat anti-rabbit IgG (1:200) for 60 min at room temperature. c-Fos was then visualized using the VECTASTAIN™ Elite ABC kit (Vector Laboratories, Burlingame, CA) for 30 min with a 5 min exposure to SIGMAFAST™ 3,3'-diaminobenzidine with metal enhancer (Sigma, St. Louis, MO). After rinsing in 0.01 M phosphate buffered saline, sections were incubated in horse anti-mouse IgG (1:200) for 60 min at room temperature, and then DBH was visualized using the ABC kit for 30 min and diaminobenzidine for 5 min. Finally, slides were cover-slipped. In control sections, normal rabbit serum substituted for primary antibody.

Microscopy and image analysis

Anatomically matched coronal sections of the NTS-A2 and -C2 region from each cohort were analyzed by light microscopy at 20 $\times$ (Olympus BX60, Olympus America Inc., Center Valley, PA), using the central canal or 4th ventricle to standardize the anteroposterior position of each region. From each brain, 6 sections (50 μ m apart) containing the NTS-A2 and 4 sections containing the -C2 region were chosen for analysis; these sections were comparable between rats. The DBH⁺ and c-Fos⁺/DBH⁺ cells were visualized and counted by a single investigator who was blind to the identity of the treatment groups. For each rat, the mean cell number in each region was calculated from the average number of positive cells present bilaterally in all sections containing the NTS-A2 (n=6) or -C2 (n=4), respectively.

Data analysis and statistics

Chromatographic data were collected and analyzed with the PowerChrom system (ADInstruments, Castle Hill, Australia). To normalize for baseline differences and analyze the change in NE release after footshock relative to the baseline in each animal, NE levels were expressed as a percentage of the pre-shock baseline, defined as the average concentration of the 3 consecutive samples collected before footshock. The cumulative incremental change in NE levels, from baseline to post-shock levels, was calculated by application of the following formula to the data from each animal: (NE level_{10min} - baseline) + (NE level_{20min} - baseline) + --- + (NE_{70min} - baseline). Similarly, incremental change was calculated for ACTH and corticosterone. Data were expressed as mean $\pm$ SEM.

ACTH and corticosterone data from experiments 2-4 were analyzed using factorial ANOVA. NE levels, compiled lever press data from experiments 1-3, and immunocytochemical data on DBH and c-Fos were analyzed using two-way ANOVA to determine the effects of treatment in nicotine and/or saline self-administration groups. Time (shock or no shock), self-administration group (nicotine or saline), PVN treatment (drug or vehicle), and dose of phenylephrine or ACTH₁₋₂₄ were treated as fixed factors in all ANOVAs. Statistical significance was assigned at $p < 0.05$.

Results

Nicotine self-administration

Adult male Sprague-Dawley rats were given *ad lib* access to nicotine for 23 hours/day and acquired nicotine self-administration without prior training, priming or food deprivation. Figure 1 represents the compiled lever press data from all rats in experiments 1-3. In rats self-administering nicotine, the number of active lever presses were approximately 3-4-fold greater than inactive presses ($F_{(1, 472)} = 650.4, p < 0.001$). Active and inactive presses were similar in rats self-administering saline. The combination of footshock and venous blood sampling did not affect active presses by rats self-administering nicotine on day 12 (63.6 ± 8.0 vs. 55.7 ± 4.7 for self-administration day 11 vs. day 12, $p > 0.05$). Total daily nicotine intakes on the days before test day 11 and 14 were: 1.12 ± 0.1 and 1.13 ± 0.1 mg/kg/day, respectively; these intakes were attributable to approximately 38 infusions per day.

PVN NE responses to mild footshock stress

In the experiment 1, the effect of footshock on the PVN NE levels was determined by *in vivo* microdialysis on day 12 of nicotine self-administration. The location of the membrane segment of microdialysis probes situated within the PVN of all rats, which were included in the data analysis, is shown in supplemental figure 1A. Based on these probe placements relative to the dimensions of the PVN, individual dialysates reflect NE throughout the PVN.

Mild footshock stress increased the PVN NE release in both rats self-administering nicotine and saline (Figure 2A: time, $F_{(9, 200)} = 13.8, p < 0.001$). NE levels were elevated for a longer duration in rats self-administering saline (30 min) than nicotine (20 min; $p < 0.05$), and nicotine self-administration reduced the magnitude of the NE response (self-administration group, $F_{(1, 200)} = 21.3, p < 0.001$; time $\times$ self-administration group, $F_{(9, 200)} = 3.1, p < 0.01$). In figure 2B, the cumulative (across time) incremental NE response was 2.5-fold less in rats self-administering nicotine than saline ($p < 0.05$). Nicotine self-administration did not affect basal NE levels in the PVN (nicotine vs. saline self-administration: 1.13 ± 0.13 vs. 1.03 ± 0.18 pg/20 μ l; $p > 0.05$). Therefore, the footshock-induced release of PVN NE was markedly reduced by nicotine self-administration.

Effects of the PVN α_1 adrenergic receptors blockade on ACTH and corticosterone secretion induced by mild footshock stress

To evaluate the role of PVN NE in the enhanced ACTH and corticosterone responses to stress observed during nicotine self-administration, the α_1 adrenergic receptor antagonist, prazosin (0.5 nmol/side), was bilaterally microinjected into the PVN 10 min before footshock. The schematic representation of the prazosin microinjection sites within the PVN is shown in supplemental figure 1B.

Figure 3 shows the effects of prazosin microinjection into the PVN on ACTH and corticosterone secretion induced by footshock during nicotine and saline self-administration. Mild footshock stress stimulated both ACTH (panel A: time, $F_{(4, 90)} = 57.9, p < 0.001$), and corticosterone release (panel B: time, $F_{(4, 70)} = 123.2, p < 0.001$). Both ACTH and

corticosterone were increased to a greater extent in rats self-administering nicotine (self-administration group, $F_{(1,90)} = 6.2$ and $F_{(1,70)} = 4.6$ for ACTH and corticosterone, respectively, $p < 0.05$). Prazosin reduced both ACTH and corticosterone responses in both rats self-administering nicotine and saline to identical levels [for ACTH: PVN treatment (prazosin vs. vehicle), $F_{(1,90)} = 20.7$, $p < 0.001$; time $\times$ PVN treatment, $F_{(4,90)} = 11$, $p < 0.001$; for corticosterone: PVN treatment, $F_{(1,70)} = 24.7$, $p < 0.001$; time $\times$ PVN treatment $F_{(4,70)} = 5.8$, $p < 0.05$], Panels C and D show the cumulative reductions (i.e., decremental response) in footshock-induced ACTH and corticosterone induced by prazosin. In rats self-administering nicotine, the prazosin-induced ACTH and corticosterone decrements were approximately 2-fold greater than in rats self-administering saline (ACTH: -106 ± 14 vs. -62.8 ± 6.4 pg/ml, $p < 0.05$; corticosterone: -323.1 ± 68.6 vs. -160.3 ± 36.2 ng/ml, $p < 0.05$). Therefore, both ACTH and corticosterone responses to footshock are more dependent on adrenergic drive during nicotine self-administration, despite the fact that significantly less NE is released in this group.

Activation of the PVN α_1 adrenergic receptors induces ACTH and corticosterone secretion

To determine whether the PVN neurons are intrinsically more sensitive to NE during nicotine self-administration, the α_1 adrenergic receptor agonist, phenylephrine (10, 40 nmol/side), was bilateral microinjected into the PVN. The distribution of intra-PVN microinjection sites (not shown) was similar to that shown for prazosin. Figure 4 shows that microinjection of 10 and 40 nmol phenylephrine stimulated both ACTH (Panel A: time, $F_{(4,100)} = 17.8$, $p < 0.001$; dose of phenylephrine, $F_{(2,100)} = 6.4$, $p < 0.01$) and corticosterone secretion (Panel B: time, $F_{(4,100)} = 47.9$, $p < 0.001$; dose of phenylephrine, $F_{(2,100)} = 33.2$, $p < 0.01$) in both rats self-administering nicotine and saline. However, nicotine self-administration did not modify the ACTH and corticosterone responses to either dose of phenylephrine (self-administration group, $F_{(1,100)} = 0.02$ and 0.5 , respectively, $p > 0.05$). Panels C and D show the cumulative incremental responses of ACTH and corticosterone to phenylephrine; these were unaffected by nicotine self-administration. Therefore, the intrinsic responsiveness of the PVN neurons to NE is not modulated by nicotine self-administration in the absence of stress.

Adrenal responsiveness to ACTH₁₋₂₄ injection

To determine whether adrenal responsiveness to ACTH is altered by nicotine self-administration, ACTH₁₋₂₄ (15 and 300 ng/kg) or vehicle was i.v. administered. Figure 5A shows that injection of 15 and 300 ng/kg ACTH₁₋₂₄ significantly increased corticosterone secretion in both rats self-administering nicotine and saline. (time, $F_{(4,90)} = 216$, $p < 0.001$; dose of ACTH, $F_{(2,90)} = 253$, $p < 0.001$; time $\times$ ACTH, $F_{(8,90)} = 59$, $p < 0.001$). Nicotine self-administration did not alter the corticosterone response to ACTH (self-administration group, $F_{(1,90)} = 0.2$, $p > 0.05$). Panel B shows that cumulative incremental corticosterone responses to both doses of ACTH were unaffected by nicotine self-administration. Therefore, the adrenal responsiveness to ACTH is not modulated by nicotine self-administration.

Induction of c-Fos in nucleus of the solitary tract (NTS)-A2 and -C2 catecholaminergic neurons by mild footshock stress

Photomicrographic images of c-Fos induction by footshock in the NTS-A2 and -C2 catecholaminergic neurons (DBH⁺) are presented in figure 6. In both rats self-administering nicotine and saline, mild footshock stress induced c-Fos expression in the NTS-A2 and -C2 DBH⁺ neurons (Figure 6A-D). In animals self-administering saline, a large number of neurons were double-labeled for c-Fos⁺ and DBH⁺ in both regions of the NTS (Figure 6A, B, and E, G). In animals self-administering nicotine, fewer DBH⁺ neurons expressed c-Fos in the NTS-A2 and -C2 (Figure 6C, D and F, H).

Figure 7 shows the semi-quantitative analysis of c-Fos expression. Nicotine SA and/or footshock did not affect the number of DBH⁺ neurons in the NTS-A2 and -C2. Nicotine self-administration diminished the number of c-Fos⁺/DBH⁺ neurons induced by footshock in the NTS-C2 (panel A: shock, $F_{(1,12)} = 47$, $p < 0.001$; self-administration group, $F_{(1,12)} = 5$, $p < 0.05$; self-administration group $\times$ shock, $F_{(1,12)} = 7.6$, $p < 0.05$), but not in the NTS-A2 (shock, $F_{(1,12)} = 58.6$, $p < 0.001$; self-administration group, $F_{(1,12)} = 2.2$, $p > 0.05$). Panel B shows the percentage of DBH⁺ neurons that were positive for c-Fos. Nicotine self-administration attenuated the increase in the percentage of DBH⁺ neurons that expressed c-Fos induced by footshock (from 10-15% basal level to >80%) in the NTS-A2 (self-administration group, $F_{(1,12)} = 4.9$, $p < 0.05$; self-administration group $\times$ shock, $F_{(1,12)} = 14.8$, $p < 0.01$), and -C2 (self-administration group, $F_{(1,12)} = 26$, $p < 0.001$; self-administration group $\times$ shock, $F_{(1,12)} = 34.5$, $p < 0.001$). In summary, nicotine self-administration reduced both the number and the fraction of the NTS catecholaminergic neurons that were activated by stress.

Discussion

Despite the enhanced HPA response to mild footshock stress seen in animals self-administering nicotine (Chen et al. 2008), the current experiments demonstrate that chronic nicotine self-administration attenuated both footshock-induced NE secretion within the PVN and c-Fos expression in the catecholaminergic neurons of the NTS-A2/C2 regions, which innervate the pcPVN (Cunningham and Sawchenko 1988, Cunningham *et al.* 1990). The impact of this alteration in the PVN NE neurotransmission on noradrenergic regulation of the HPA axis was determined in pharmacological studies. Blockade of the PVN α_1 adrenergic receptors diminished the augmentation of ACTH and corticosterone responses to mild footshock stress in rats self-administering nicotine, and the decrement of footshock-induced ACTH and corticosterone secretion was significantly greater in rats self-administering nicotine than saline. Nevertheless, in the absence of footshock, activation of the PVN α_1 adrenergic receptors stimulated ACTH and corticosterone release to a similar degree in both self-administration groups. Therefore, the footshock-induced NTS activation and PVN NE input are both attenuated by nicotine self-administration, yet the PVN CRF neurons are more responsive to α_1 adrenergic stimulation, but only during footshock stress. During footshock, this enhanced noradrenergic response of the PVN CRF neurons is necessary for the augmented HPA response in animals self-administering nicotine.

The PVN NE secretion, induced by various stressors, is known to regulate the pcPVN CRH neurons and HPA axis (Pacak et al. 1995a). Acute stressors, such as mild footshock, immobilization, cold, and subcutaneous injection of formalin, increased the release of PVN NE (Pacak *et al.* 1995b, Ishizuka *et al.* 2000). Previous studies also have shown that the duration, quality and severity of a chronic stressor affects the subsequent response of PVN NE to an acute stressor, with both enhancement and attenuation reported (Stanford 1995). In the current studies, we found that footshock increased the PVN NE levels for 30min in rats self-administering saline. In comparison, nicotine self-administration reduced the magnitude and duration of the PVN NE response to footshock. Since the pcPVN NE inputs originate from catecholaminergic neurons within the NTS-A2/C2 regions (Cunningham and Sawchenko 1988, Cunningham *et al.* 1990), the decreased activation of the NTS catecholaminergic neurons, evident in the reduction of footshock-induced c-Fos expression by the NTS-A2/C2 neurons in rats self-administering nicotine, may underlie the diminished the PVN NE response to footshock during nicotine self-administration.

The PVN NE inputs, originating in the NTS, primarily activate α_1 adrenergic receptors that are highly expressed by the pcPVN (Cummings and Seybold 1988) CRF neurons (Day et al. 1999, Sands and Morilak 1999). Studies have confirmed the critical role of the PVN

noradrenergic circuit by depleting PVN NE or administering an α_1 adrenergic antagonist, such as prazosin. Both approaches inhibited restraint or swim stress-induced corticosterone or ACTH release, respectively (Gibson et al. 1986, Toufexis and Walker 1996, Toufexis *et al.* 1998). These findings accord with the current studies, in which an intra-PVN microinjection of prazosin attenuated the ACTH and corticosterone response to footshock in rats self-administering saline. Indeed, the reduction of footshock-induced ACTH and corticosterone secretion due to prazosin was significantly greater (2-fold) during nicotine self-administration, which itself acts as a chronic stressor (Chen et al. 2008, Yu et al. 2008). Consistent with our studies of nicotine self-administration, chronic intermittent exposure to cold enhanced the ACTH response to immobilization, although PVN NE secretion was not augmented (Ma and Morilak 2005). Intra-PVN microinjection of benoxathian, an α_1 adrenergic antagonist, blocked this enhanced ACTH release, despite the fact that it was not accompanied by a greater secretion of PVN NE than in control rats without chronic cold exposure. Therefore, after chronic exposure to a stressor (e.g., nicotine self-administration, intermittent cold), the corticosterone response to a heterologous stressor is augmented, depending in part on activation of α_1 adrenergic receptors by NE. However, the acute stress-induced secretion of NE is reduced or unchanged, depending on the chronic stressor.

In rats self-administering nicotine, the increased efficacy of prazosin in reducing footshock-induced corticosterone indicates that the hypophysiotropic pcPVN CRF neurons may be more responsive to noradrenergic stimulation. This could also be evaluated by testing the efficacy of a lower dose of prazosin to attenuate the HPA response to stress in rats self-administering nicotine but not saline. Noradrenergic responsiveness was further evaluated by intra-PVN microinjections of phenylephrine, an agonist specific for α_1 adrenergic receptors. We found that, in the absence of stress, the intrinsic HPA responsiveness to α_1 adrenergic stimulation of PVN was unaffected by nicotine self-administration. Additionally, the adrenal responsiveness to stimulation by ACTH was unaffected by nicotine self-administration. In contrast, chronic cold exposure enhanced HPA responsiveness to intra-PVN phenylephrine (Ma and Morilak 2005). Thus, different chronic stressors induce specific changes in PVN adrenergic responsiveness to an acute stressor. Our data does not provide evidence supporting the enhanced expression or function of the PVN α_1 adrenergic receptors during nicotine self-administration.

Since GABA is involved in regulating the HPA response to stress (Herman *et al.* 2004), it is conceivable that nicotine self-administration may alter its release or action during acute stress. Previous studies, showing that NE modulates GABAergic inputs to the pcPVN, provide evidence in support of this postulate. Through α_1 adrenergic receptors, NE increased inhibitory synaptic currents in 59% of pcPVN neurons (Daftary et al. 2000). Therefore, in rats self-administering nicotine during mild footshock stress, inhibitory regulation of CRF neurons might be lessened due to the reduction in NE release and, consequently, in GABA secretion. The resulting disinhibition of CRF neurons would potentiate their responsiveness to NE.

The analgesic effects of nicotine are not likely to account for the effects of nicotine self-administration on the HPA response to mild footshock stress in the models used in these studies. The analgesic effects of a chronic high dose infusion of nicotine (6mg/kg/day) were previously demonstrated in tail flick and hot-plate tests (Carstens *et al.* 2001). Chronic exposure of rats to a very high level of cigarette smoke, resulting in venous plasma nicotine levels of 100 ng/ml, also induced analgesia in the tail flick test (Anderson *et al.* 2004). In our study, rats received only 1.1 mg/kg /day of nicotine, an amount similar to that obtained by moderate human smokers (Valentine et al. 1997), and a dose considerably less than that required for significant analgesia. Rather than attenuating the HPA response to stress, the current study and a previous report (Chen et al. 2008) show that nicotine enhanced this

response. This would not be the expected outcome were nicotine acting primarily as an analgesic agent. Lastly, the very mild footshock (0.6 mA, 0.5s) applied in this study has been shown to induce emotional stress by means other than physical pain (Ripoll *et al.* 2006).

We have previously shown that chronic nicotine self-administration functions as a stressor by inducing the expression of CRF⁺/AVP⁺ neurons in the pcPVN, which are activated by footshock, thereby contributing to the enhanced HPA responsiveness to footshock (Chen *et al.* 2008, Yu *et al.* 2008). The current study demonstrates that chronic nicotine self-administration diminished both footshock-induced activation of the NTS and the PVN NE secretion, yet the PVN CRF neurons were more responsive to α_1 adrenergic stimulation, but only during mild footshock stress. This plasticity in noradrenergic regulation of the PVN CRF neurons provides a new mechanism that contributes to the HPA sensitization to stress induced by chronic nicotine self-administration and smoking.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ACTH	adrenocorticotrophic hormone
AVP	arginine vasopressin
CRF	corticotropin-releasing factor
DBH	dopamine β -hydroxylase
HPA	hypothalamo-pituitary-adrenal
NE	norepinephrine
NTS	nucleus of the solitary tract
PVN	hypothalamic paraventricular nucleus
pcPVN	parvocellular division of the PVN

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Fig. 1.

The acquisition and maintenance of nicotine (Nic) self-administration (SA) in adult male Sprague-Dawley rats without prior training, priming or food deprivation. Nicotine (30 μ g/kg, i.v. injection) was available 23 hours/day for 12-15 days, terminating on the 15th day in microinjection experiments. Nic SA rats had significantly more active than inactive lever presses [two way ANOVA: time, $p < 0.001$; SA (active vs. inactive), $p < 0.001$; time $\times$ SA, $p < 0.001$] and more active presses than the saline (Sal) SA group (two way ANOVA: time, $p < 0.001$; SA group, $p < 0.001$; time $\times$ SA group, $p < 0.001$). Active and inactive presses were not different in Sal SA [time, $p > 0.05$, SA (active vs. inactive), $p > 0.05$]. All data are expressed as mean $\pm$ SEM.

Fig. 2.

The effects of nicotine self-administration (SA) on the PVN norepinephrine (NE) secretion (panel **A**) and incremental NE response (**B**) to mild footshock stress. NE was measured by *in vivo* microdialysis and HPLC-electrochemical detection; NE levels were expressed as a percentage of the pre-footshock baseline levels. Panel **A**: Nicotine SA significantly attenuated the PVN NE response to footshock compared to saline SA (two way ANOVA: time, $p < 0.001$; SA group, $p < 0.001$; time $\times$ SA group, $p < 0.01$). Basal NE levels for nicotine and saline SA: 1.13 ± 0.13 vs. 1.03 ± 0.18 pg/20 μ l, respectively, were not significantly different ($p > 0.05$; *t*-test). *, $p < 0.05$, vs 3 pre-shock baseline levels, respectively; #, $p < 0.05$, saline vs nicotine SA at same time point, respectively. Panel **B**: The cumulative (across time) incremental NE response to footshock was less in nicotine SA than saline SA rats. **, $p < 0.01$, nicotine vs saline SA, (*t*-test). All data are expressed as mean $\pm$ SEM.

Fig. 3.

The effects of prazosin on ACTH (panel **A, C**) and corticosterone levels (**B, D**) induced by mild footshock stress during nicotine (Nic) or saline (Sal) self-administration (SA). Prazosin (Praz; α_1 adrenergic receptor antagonist; 0.5 nmol/side) or vehicle (Veh) was microinjected bilaterally into the PVN 10 min prior to footshock. Panel **A**: Plasma ACTH levels were increased by footshock (factorial ANOVA: time, $p < 0.001$) and the response was greater in nicotine SA rats (SA group, $p < 0.05$). Prazosin attenuated footshock-induced ACTH release in both Nic SA and Sal groups (PVN treatment, $p < 0.001$; time $\times$ PVN treatment $p < 0.001$). Panel **B**: Plasma corticosterone levels were increased by footshock (factorial ANOVA: time, $p < 0.001$), and the response was greater in nicotine SA rats (SA group, $p < 0.05$). Prazosin attenuated footshock-induced corticosterone release in both Nic SA and Sal groups (PVN treatment, $p < 0.001$; time $\times$ PVN treatment, $p < 0.001$). Panels **C** and **D**: In the nicotine SA group, prazosin induced a significantly greater cumulative (across time) decrement in footshock-induced ACTH and corticosterone secretion than in saline SA rats. *, $p < 0.05$, nicotine vs. saline SA (t -test).

Fig. 4.

The effects of phenylephrine on plasma ACTH (panel **A, C**) and corticosterone (**B, D**) levels during nicotine (Nic) or saline (Sal) self-administration (SA). Phenylephrine (PE; α_1 adrenergic agonist, 10 and 40 nmol/side) or vehicle was bilaterally microinjected into the PVN 10 min prior to footshock. Panel **A**: Both 10 and 40 nmol PE significantly increased ACTH secretion (factorial ANOVA: time, $p < 0.001$; dose of PE, $p < 0.01$; time $\times$ PE, $p < 0.05$). Nicotine SA did not affect the ACTH response to PE (SA group, $p > 0.05$). Panel **B**: Both 10 and 40 nmol PE significantly increased corticosterone secretion (factorial ANOVA: time, $p < 0.001$; dose of PE, $p < 0.01$; time $\times$ PE, $p < 0.05$). Nicotine SA did not affect the corticosterone response to PE (SA group, $p > 0.05$). Panels **C** and **D**: Incremental ACTH and corticosterone secretion was increased to the same extent in Nic SA and Sal groups, although the incremental ACTH and corticosterone secretion induced by PE 40 nmol was significantly higher than that by PE 10 nmol in both Nic SA and Sal groups *, $p < 0.05$, PE 40 vs.10 nmol, respectively (t -test).

Fig. 5.

The effects of ACTH₁₋₂₄ on plasma corticosterone levels (panel **A**) and the incremental corticosterone response (**B**) during nicotine (Nic) or saline (Sal) self-administration (SA). ACTH₁₋₂₄ (15 and 300 ng/kg) or vehicle was injected i.v. Panel **A**: Both doses of ACTH significantly increased corticosterone secretion (factorial ANOVA: time, $p < 0.001$; dose of ACTH, $p < 0.001$; time $\times$ ACTH, $p < 0.001$). Nicotine SA did not affect the corticosterone response to ACTH (SA group, $p > 0.05$). Panel **B**: Incremental corticosterone secretion was increased to the same extent in Nic SA and Sal groups, although the response to ACTH 300 ng/kg was significantly greater than to ACTH 15 ng/kg in both Nic SA and Sal groups *, $p < 0.05$, ACTH vs. vehicle, respectively; # $p < 0.05$, ACTH 300 vs 15 ng/kg respectively (t -test).

Fig. 6.

Activation of the nucleus of the solitary tract (NTS)-A2 and -C2 catecholaminergic neurons by mild footshock stress in rats during nicotine vs. saline self-administration (SA). Photomicrographs illustrate immunocytochemical detection of dopamine β -hydroxylase (DBH; brown) and c-Fos (black) within the NTS-A2 and -C2 regions of representative tissue sections on SA day 12. The upper and middle rows show the coronal NTS-A2 and -C2 profiles of DBH⁺ neurons in saline SA (panel **A, B**) and nicotine SA (**C, D**) rats [10 $\times$ magnification; central canal (cc); 4th ventricle (IV); insets indicate the positions of the 40 $\times$ images shown in **E-H**]. In saline SA rats, footshock induced the expression of c-Fos in the NTS A2 (**E**) and C2 (**G**), and a large number of DBH⁺ neurons were double-labeled for c-Fos (arrowhead, DBH⁺ neurons; arrow, Fos⁺/DBH⁺). In nicotine SA rats, fewer Fos⁺/DBH⁺ neurons were activated by footshock in the NTS-A2 (**F**) and -C2 (**H**) than in saline SA rats. Scale bars: **A-D**, 50 μ m; **E-H**, 20 μ m.

Fig. 7.

Nicotine self-administration (SA) decreased the number (panel **A**) and percentage (**B**) of neurons activated in the NTS-A2 and -C2 regions by mild footshock stress. Panel **A**: Nicotine SA and/or footshock did not affect the number of DBH⁺ neurons in the NTS-A2 and -C2 (two way ANOVA: SA group in the NTS-A2 and -C2, $p > 0.05$, respectively; shock in A2 and C2, $p > 0.05$, respectively). Nicotine SA significantly reduced the number of the NTS-C2 catecholaminergic neurons expressing c-Fos (Fos⁺/DBH⁺) induced by footshock (shock, $p < 0.001$; SA group, $p < 0.05$; SA group $\times$ shock, $p < 0.05$), but not in the NTS-A2 (shock, $p < 0.001$; SA group, $p > 0.05$). * $p < 0.05$, nicotine SA + shock vs. saline SA + shock (t -test). Panel **B**: Nicotine SA reduced the percentage of catecholaminergic neurons activated by mFSS (expressed as the percentage of all DBH⁺ neurons that were also c-Fos⁺) within both the NTS-A2 (two-way ANOVA: SA group, $p < 0.05$; shock, $p < 0.001$; SA group $\times$ shock, $p < 0.01$) and -C2 regions (SA group, $p < 0.05$; shock, $p < 0.001$; SA group $\times$ shock, $p < 0.001$). * $p < 0.05$, nicotine SA + shock vs. saline SA + shock, respectively (t -test).