JPET #112474

Nicotine-induced norepinephrine release in hypothalamic paraventricular nucleus and amygdala is mediated by NMDA receptors and nitric oxide in the nucleus tractus solitarius

Rongjie Zhao, Hao Chen and Burt M. Sharp

Department of Pharmacology, University of Tennessee Health Science Center,

Memphis, TN 38163 USA

1

Running title: nicotine, norepinephrine, NMDA receptors, NO and NTS

Corresponding author:

Burt M. Sharp, M.D.

Department of Pharmacology, University of Tennessee Health Science Center, 874 Union

Avenue, Memphis TN 38163; Phone: 901-448-6000; FAX 901-448-7206; E-mail:

bsharp@utmem.edu

Section: Neuropharmacology

Number of pages: 32

Number of figures: 6

Number of tables: 0

Number of references: 40

Number of words: 249 in Abstract

659 in Introduction

1184 in Discussion

Abbreviations: NE, norepinephrine; NO, nitric oxide; NTS, nucleus tractus solitarius;

AP-5, DL-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2, 3-

dione; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-

oxide; L-NAME, NG-nitro-L-arginine methyl ester hydrochloride; PVN, hypothalamic paraventricular nucleus; AMYG, amygdala; pcPVN, parvocellular PVN; CRH, corticotropin releasing-hormone; nicotinic cholinergic receptor, nAChR; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; NOS, nitric oxide synthase; KRB, Kreb's Ringer Buffer; LC, locus coeruleus.

Abstract

The noradrenergic projections from brainstem nucleus tractus solitarius (NTS) to hypothalamic paraventricular nucleus (PVN) and amygdala (AMYG) are involved in nicotine-related stress responses and drug craving. Previous studies demonstrated that i.v. nicotine-induced norepinephrine (NE) release in the PVN and AMYG depends on nicotinic cholinergic receptors in the brainstem. However, the direct site and mechanism of nicotine's action in brainstem are unknown. The present study determined the roles of NTS ionotropic glutamate receptors and nitric oxide (NO) in the effects of both local and systemic nicotine on NE release in PVN and AMYG. In male rats, an intra-NTS microinjection of nicotine (1.2 µg, free base) or i.v. nicotine infusion (0.065 or 0.09 mg/kg) significantly increased NE levels in PVN and AMYG microdialysates. Prior microinjection of the NMDA receptor antagonist, DL-2-amino-5-phosphonopentanoic acid (AP–5; 0.75 or 1.5 µg), but not an AMPA receptor antagonist, dose-dependently nearly abolished both PVN and AMYG NE responses to nicotine administered into NTS or systemically. NO involvement was assessed with intra-NTS microinjections of the nonselective NOS inhibitor, NG-nitro-L-arginine methyl ester hydrochloride (L–NAME, 10-30 nmol), or the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO, 0.1-0.2 nmol); both agents dose-dependently inhibited i.v. nicotine-induced NE release. These results indicate that nicotine–induced NE release in PVN and AMYG is mediated entirely through the local effects of nicotine on NTS glutamate afferents and NMDA receptors that, in part, stimulate NO production, resulting in activation of noradrenergic neurons. Therefore, nicotine acts indirectly on noradrenergic NTS neurons to elicit NE release in forebrain structures.

Introduction

Nicotine, the principal psychoactive component of tobacco, stimulates noradrenergic pathways that affect neurotransmission in diverse regions within the central nervous system. These include the hypothalamic paraventricular nucleus (PVN) and amygdala (AMYG), considered central to stress responsiveness or drug craving, respectively (Fu et al., 1997; Matta et al., 1998; Dani and De Biasi, 2001). By activating nicotinic cholinergic receptors (nAChRs), nicotine evokes norepinephrine (NE) release in PVN and AMYG from fibers originating in the brainstem nucleus tractus solitarius (NTS) (Fu et al., 1997; Fu et al., 1998).

NE is one of the primary neurotransmitters mediating the PVN response to stressors (Matta et al., 1998). The parvocellular PVN (pcPVN) converts neuronal signals into endocrine output by secreting corticotropin releasing-hormone (CRH), which, in turn, stimulates ACTH secretion from the pituitary. Previous studies have demonstrated that nicotine administered into the fourth cerebroventricle or by way of an i.v. infusion stimulates NE release and c-Fos expression in the pcPVN (Valentine et al., 1996; Matta et al., 1998). These are dose-dependently correlated with nicotine-stimulated ACTH secretion, largely through recruitment of the noradrenergic system projecting from the NTS-A2 region to pcPVN (Matta et al., 1993; Fu et al., 1997).

We also have reported that NE is released into AMYG in rats during chronic nicotine self-administration (Fu et al., 2003). The AMYG is involved in emotional memory (Cahill et al., 1996), working memory performance (Ohno et al., 1993), and the

regulation of memory storage in other brain regions (Galvez et al., 1996). In addition, the AMYG is essential for the acquisition of conditioned reinforcement, (Arroyo et al., 1998). Hence, rats with bilateral AMYG lesions do not acquire drug associated (i.e. cocaine) cue-dependent behavior in which an environmental cue acquires some of the properties of the primary drug reinforcer (Arroyo et al., 1998). Multiple studies have implicated NE in memory consolidation by the AMYG (Galvez et al., 1996; Ferry et al., 1999; Hatfield et al., 1999; McGaugh and Izquierdo, 2000). An infusion of NE into the AMYG enhanced memory consolidation, whereas local injection of α- or β-adrenergic receptor antagonists inhibited the memory enhancing effects of epinephrine (Ferry et al., 1999; Hatfield et al., 1999; McGaugh and Izquierdo, 2000). Additionally, memory retention in the absence or presence of contextual cues was enhanced by the release of NE from NTS noradrenergic projections to AMYG (Williams et al., 2000).

The NTS integrates both visceral and external environmental information, signaling the PVN and limbic areas such as AMYG (Boscan et al., 2002; Buller, 2003). nAChRs expressed in NTS (Ashworth-Preece et al., 1998) have been shown to be involved in the modulation of evoked release of excitatory amino acids in NTS (Ashworth-Preece et al., 1998). Moreover, nicotine elicited glutamate release in NTS and other regions such as prefrontal cortex and nucleus accumbens, (Reid et al., 2000; Lambe et al., 2003). Both α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and N-methyl-D-aspartate (NMDA) receptors are present in NTS (Zhang and Mifflin, 1998). Moreover, NMDA receptors and neuronal nitric oxide synthase (nNOS) co-localize within NTS neurons (Lin and Talman, 2000). NO has been shown to enhance neurotransmitter release

in many brain regions, including NE in hippocampus (Lonart et al., 1992). Therefore, we hypothesized that nicotine stimulates NTS glutamate secretion, activating NMDA receptors and inducing NO release, which, in turn, stimulates NE neurons projecting to PVN and AMYG.

In this investigation, NE levels in both PVN and AMYG were measured simultaneously by *in vivo* microdialysis and antagonists were administered by microinjection directly into the NTS-A2. To assess the role of glutamate, characterize the type of ionotropic glutamate receptor involved, and determine whether systemic and local nicotine act directly through the NTS, we microinjected NMDA or AMPA receptor antagonists (AP-5 and CNQX respectively) into NTS and then administered nicotine directly into NTS or by an i.v. infusion. To determine the involvement of NTS NO in nicotine-induced NE release in forebrain structures, intra-NTS microinjections of the nonselective NOS inhibitor, L-NAME, or the NO scavenger, carboxy-PTIO, were administered prior to i.v. nicotine.

Methods

Materials

(-)-Nicotine hydrogen tartrate (all doses expressed as free base), pH 7.2, norepinephrine (NE) hydrochloride, nomifensine maleate, DL-2-amino-5-phosphonopentanoic acid (AP-5), 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX), NG-nitro-L-arginine methyl ester hydrochloride (L-NAME) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium dihydrogen phosphate monohydrate, EDTA, 1-decanesulfonic acid, acetonitrile, methanol, and phosphoric acid were from Fisher Scientific Co. (Fair Lawn, NJ). Cellulose fiber tubing was purchased from Spectrum (Laguna Hills, CA), and silica tubing (outer diameter, 148 µm; inner diameter, 73 µm; TSP 075150) from Polymicron Technologies Inc. (Phoenix, AZ).

Animals

All procedures were conducted in accordance with NIH Guidelines concerning the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center. Adult male Sprague-Dawley rats (300 - 330 g, Harlan, Madison, WI) were given access *ad libitum* to standard rat chow and water. Rats were individually housed on a 12-h reversed light cycle (off at 9 A.M , on at 9 P.M.) for 14 d prior to microdialysis experiments. After 7 d of housing under these conditions, rats were anesthetized with xylazine-ketamine (5:35 mg/kg b.wt., i.m.; Sigma-Aldrich), and chronic guide cannulae were stereotaxically implanted into the

PVN, AMYG and NTS, according to the coordinates of Paxinos and Watson (Paxinos and Watson, 1986). Coordinates from bregma with flat skull were: PVN, AP -2.0 mm, DV -7.4 mm, ML 0.3 mm; AMYG, AP -2.7 mm, DV -7.5 mm, ML 4.2 mm; NTS, AP -13.6 mm, DV -6.5 mm, ML -0.8 mm. 5 d thereafter, the jugular vein was cannulated under xylazine-ketamine; animals were recovered for 2 d. 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.

In vivo microdialysis

Concentric microdialysis probes (1.5 mm for PVN, 2 mm for AMYG; MW cutoff 13,000 Da, outer diameter 235 µm) were constructed in our laboratory, as reported previously (Fu et al., 1997). The recovery rate of individual probes was determined by *in vitro* dialysis of a solution containing 19.45 nM NE for 45 min at 22 °C. Triplicate 15-min samples were obtained, and the recovery rate was 5.8% $\pm$ 0.6 for the 1.5 mm probe and 9.8% $\pm$ 1.2 for the 2 mm probe (n=20 for each size probe).

Microdialysis was performed as described previously (Fu et al., 1997). Briefly, on the day of microdialysis, during their active (dark) phase, rats were moved into the alert-rat microdialysis chambers (CMA, Chelmsford, MA) within an isolated dark room, lit by a red safe-light; microdialysis probes were inserted into both PVN and AMYG guide cannulae, and an injection needle was inserted into the NTS guide cannula. Following insertion, probes were perfused (1.5 µl/min) with Kreb's Ringer Buffer (KRB; 147 mM NaCl, 4.0 mM KCl, and 3.4 mM $CaCl_2$ in polished water; 0.2 µm filter sterilized and

degassed) containing 5 µM nomifensine (selected to inhibit NE reuptake in order to permit internal comparison of basal and peak NE levels to previous studies from our laboratory) for 2 h. Thereafter, 15-min microdialysate samples were collected into glass vials containing 1 µl of 5% perchloric acid; three consecutive samples were collected to measure basal NE levels prior to drug administration.

HPLC and electrochemical analysis

Samples (15 µl) were injected by a CMA 200 refrigerated autosampler onto a 150 x 2 mm ODS C18 column (ESA, Chelmsford, MA) perfused (0.25 ml/min; ESA model 582 pump) by mobile phase containing 50 mM sodium dihydrogen phosphate monohydrate, 0.7 mM EDTA, 2 mM 1-decanesulfonic acid, 11% methanol, and 11% acetonitrile, pH 6.0. Electrochemical detection at 220 mV and 1.0 nA was performed with an ESA Coulochem II 5200A equipped with an ESA 5041 high-sensitivity microbore analytical cell. The limit of detection for NE was 100 fg/injection. Previous reports from our laboratory have demonstrated representative chromatograms (Fu et al., 1998).

Experimental protocols

The first experiment was performed to determine whether NMDA or AMPA receptors in NTS mediate the release of NE in PVN and AMYG that is stimulated by microinjecting nicotine into NTS. This was accomplished using the NMDA receptor antagonist, AP-5, and the AMPA receptor antagonist, CNQX. All animals were randomized among the various treatment groups in this and all subsequent experiments. On the day of

microdialysis, after three basal samples were obtained from PVN and AMYG, KRB, AP-5 (0.75 or 1.5 µg) or CNQX (1.0 or 2.0 µg) was unilaterally microinjected (100 nl over 30 sec) into NTS 5 min prior to KRB or nicotine (1.2 µg). We have determined, in our pilot study, that 2.0 µg CNQX is the largest dose that can be injected into NTS without inducing abnormal behavior. This dose of nicotine was chosen based on pilot studies showing robust NE release within PVN and AMYG, similar to levels attained by the dosages of i.v. nicotine used herein, and in the absence of behavioral changes. Since previous studies have shown that plasma ACTH levels are highly responsive to microinjection of nicotine into NTS-A2, this region of NTS was targeted in the present experiments (Matta et al., 1993). We previously microinjected ^{3}H-nicotine into the NTS-A2; 80% of the radial spread was within approximately 500 µm of the microinjection site and 90% was within 650 µm (Matta et al., 1993). Although our current microinjection site for NTS-A2 is approximately 800 µm posterior to the center of NTS-C2, it is very likely that the caudal area of NTS-C2 will be infiltrated by nicotine following a microinjection into NTS-A2. Therefore, in the current studies the locus of all microinjections is referred to as NTS. All drugs were dissolved in KRB and microinjected in 100 nl. The second experiment assessed whether the release of NE in the PVN and AMYG in response to systemic nicotine also depends on NTS NMDA receptors. Thus, AP-5 (1.5 µg) was microinjected unilaterally or bilaterally into NTS 5 min prior to an infusion of i.v. nicotine (0.065 or 0.09 mg/kg infused over 44 or 60 sec, respectively).

The third set of experiments were designed to investigate whether the generation of NO in NTS is involved in the release of NE in PVN and AMYG in response to systemic nicotine. The nonselective NOS inhibitor, L–NAME (10 or 30 nmol), or the NO scavenger, carboxy-PTIO (0.1 or 0.2 nmol), was microinjected bilaterally 5 min prior to an infusion of nicotine (0.065 mg/kg infused over 44 sec). Since nicotine can activate NE projections from locus coeruleus (LC) to PVN and it is known that LC is less sensitive to nicotine than NTS, in these studies we utilized 0.065 mg/kg nicotine to activate primarily NTS NE neurons.

Data analysis and statistics

Chromatographic data were collected and analyzed with the PowerChrom system (ADInstruments, Castle Hill, Australia). Data (Mean ± S.E.M.) were expressed as a percentage of the basal NE level. In each animal, the basal NE value was defined as the average level calculated from the three samples collected immediately prior to the administration of nicotine or other pharmacological agents. Peak NE levels were measured in the samples collected 15 min after nicotine administration. All data were analyzed by repeated measures two-way ANOVA (SPSS 13.0; SPSS Inc., Chicago, IL). *Post hoc* comparisons were made using the Tukey test.

Results

Histological evaluation was used to determine whether the effective membrane areas of microdialysis probes were within the PVN and central AMYG, and if the tip of microinjection cannulae resided within the NTS-A2. Fig. 1 illustrates the locations within the PVN and central AMYG of all microdialysis probes and of all microinjection cannulae within NTS-A2 that were positioned accurately.

The effects of the NMDA receptor antagonist, AP-5, on simultaneous nicotine-stimulated NE release in PVN and AMYG are shown in Fig.2, A and B. The main effects of treatment and time (ANOVA) were significant in both brain regions. Panel A shows that a unilateral NTS microinjection of nicotine (KRB/Nic 1.2 μg/100 nl/30 sec) significantly elevated NE levels in PVN compared to controls (KRB/KRB; $P < 0.01$). Within 15 min, NE levels had increased to 185 % of baseline and NE remained significantly elevated at 30 min. Pretreatment with AP-5 (AP-5/Nic) significantly and dose-dependently inhibited the NE response to nicotine (0.75 μg = 63% reduction; 1.5 μg = 100% reduction). F (treatment) = 6.08, $P < 0.01$; F (time) = 22.83, $P < 0.01$; F (time × treatment) = 11.03, $P < 0.01$. In AMYG (panel B), NE levels were elevated to a similar extent by intra-NTS microinjections of nicotine, and were significantly blocked in a dose-dependent manner by AP-5. F (treatment) = 16.48, $P < 0.01$; F (time) = 15.87, $P < 0.01$; F (time × treatment) = 9.03, $P < 0.01$. AP-5 alone [AP-5 (1.5)/KRB] did not affect NE levels in either PVN or AMYG, and basal levels (reported in the figure legends) were similar in all treatment groups.

In contrast to AP-5, the AMPA receptor antagonist, CNQX (1.0 or 2.0 µg/100 nl/30 sec), did not alter NE release in PVN and AMYG in response to a subsequent microinjection of nicotine (1.2 µg) into NTS (Fig. 3, panel A: PVN, panel B: AMYG). Additionally, CNQX alone [CNQX (2.0)/KRB] did not affect NE levels in either region. For nicotine-stimulated NE release in PVN: F (treatment) = 11.18, P < 0.01; F (time) = 47.71, P < 0.01; F (time × treatment) = 10.28, P<0.01. For AMYG: F (treatment) = 11.55, P < 0.01; F (time) = 41.40, P < 0.01; F (time × treatment) = 8.69, P < 0.01. Therefore, NMDA receptors in NTS, and not AMPA receptors, mediate nicotine-induced NE release in PVN and AMYG when nicotine is microinjected into NTS.

In Fig. 4, panels A-D, AP-5 blockade of NE release in both PVN and AMYG elicited by an i.v. infusion of nicotine showed significant main effects of treatment and time (ANOVA). Panels A and B demonstrate that the higher dosage of i.v. nicotine (0.09 mg/kg over 60 sec) resulted in a 2-fold peak increase (P < 0.01) in NE levels in PVN and AMYG, respectively. A unilateral intra-NTS microinjection of 1.5 µg AP-5 [AP-5/Nic (.09)] significantly reduced the NE response to i.v. nicotine in both PVN (panel A) and AMYG (panel B). For PVN: F (treatment) = 72.84, P < 0.01; F (time) = 81.74, P < 0.01; F (time × treatment) = 33.03, P < 0.01. For AMYG: F (treatment) = 21.99, P < 0.01; F (time) = 138.52, P < 0.01; F (time × treatment) = 47.76, P < 0.01. Panels C and D show that infusion of a lower dose of nicotine (0.065 mg/kg over 44 sec) elevated NE levels in both regions by approximately 70% above baseline (P < 0.01). A unilateral NTS microinjection of AP-5 inhibited this response by 71 %, whereas bilateral injections were more effective (approximately 90% reduction; P < 0.05 compared to unilateral AP-

14

5/Nic). For PVN: F (treatment) = 20.24, P < 0.01; F (time) = 98.62, P < 0.01; F (time × treatment) = 28.79, P < 0.01. For AMYG: F (treatment) = 10.68, P < 0.01; F (time) = 86.09, P < 0.01; F (time × treatment) = 24.39, P < 0.01. Therefore, similar to the efficacy of AP-5 at inhibiting NE responses to nicotine delivered into NTS, these results indicate that bilateral injections of AP-5 into NTS blocked almost all of the NE response to systemic nicotine.

The next series of experiments investigated the role of NO in the NE response to systemic nicotine by inhibiting NOS in NTS or sequestering NTS NO. The non-selective NOS inhibitor, L-NAME (10 or 30 nmol/100 nl/30 sec), or the NO scavenger, carboxy-PTIO (cPTIO; 0.1 or 0.2 nmol/100 nl/30 sec), were microinjected bilaterally into NTS prior to an infusion of i.v. nicotine (0.065 mg/kg over 44 sec). Significant main effects of treatment and time (ANOVA) were observed for each dose of L-NAME in both brain regions (Fig. 5). In PVN, F (treatment) = 15.26, P < 0.01; F (time) = 141.10, P < 0.01; F (time × treatment) = 22.27, P < 0.01. In AMYG, F (treatment) = 10.33, P < 0.01; F (time) = 131.56, P < 0.01; F (time × treatment) = 21.65, P < 0.01. In Fig. 5, panels A and B show that L-NAME dose-dependently inhibited i.v. nicotine-induced NE release in PVN or AMYG, respectively (P < 0.05 for each dose). In PVN, 10 nmol L-NAME inhibited the peak NE response by 52%, with 71 % inhibition by 30 nmol; a similar degree of attenuation was observed in the AMYG. Finally, as shown in Fig. 6 panels A (PVN) and B (AMYG), removal of diffusible NO with intra-NTS microinjection of the NO scavenger, carboxy-PTIO, dose-dependently attenuated nicotine-induced NE release by 42% (0.1 nmol) and 68 % (0.2 nmol) in each brain region. For PVN, F (treatment) =

30.07, P < 0.01; F(time) = 140.37, P < 0.01; F(time × treatment) = 18.51, P < 0.01. For

AMYG, F (treatment) = 11.73, P < 0.01; F (time) = 172.43, P < 0.01; F (time ×

treatment) = 22.53, P < 0.01.

Discussion

Previous studies have demonstrated that nicotine activates brainstem nAChRs accessible from the fourth ventricle, resulting in NE release in the rat PVN and AMYG (Fu et al., 1997; Fu et al., 1998). Furthermore, nicotine-stimulated ACTH secretion was shown to depend on PVN α-adrenergic receptors (Matta et al., 1990), and both PVN c-Fos expression and ACTH secretion in response to systemic nicotine were mediated through the brainstem (Matta et al., 1993; Matta et al., 1993). However, nicotine's site and mechanism of inducing NE release in PVN and AMYG are unknown. Since glutamatergic afferents to NTS are known to be activated by nicotine (Ashworth-Preece et al., 1998; Ferreira et al., 2002), the present studies focused on the role of glutamate and NO within the NTS to determine if they mediate nicotine-induced NE release in PVN and AMYG. A direct microinjection of nicotine into NTS-A2 stimulated NE release in both the PVN and AMYG within 15 min. This simultaneous NE release was dose-dependently inhibited by intra-NTS AP-5, with complete blockade by the higher dose, implicating nicotine-elicited glutamate secretion within NTS and the requisite involvement of NMDA receptors (Fig. 2). Similarly, the NE release elicited by systemic nicotine was reduced by 90% in each brain region following bilateral microinjections of AP-5 (Fig. 4). Thus, the release of NE in both PVN and AMYG by systemic nicotine depends on the same glutamatergic circuitry in NTS.

Glutamate stimulates the production of NO in many brain regions (Matsuo et al., 2001). The activation of NMDA receptors triggers an influx of Ca^{2+} into postsynaptic neurons, which then stimulates NO production by neuronal nitric oxide synthase (nNOS) (Oh,

1995). In NTS, neurons containing nNOS co-express the principal NMDA receptor subunit, NR1, although not all neurons containing NR1 co-express nNOS (Lin and Talman, 2000). Therefore, experiments were designed to determine if the observed glutamatergic mediation of nicotine-induced NE release was dependent on NO. The results demonstrate a pivotal role of NO in the NE response to systemic nicotine, as indicated by the efficacy of both a NOS inhibitor and an NO scavenger. Bilateral intra-NTS microinjection of L-NAME dose-dependently attenuated NE release, as did carboxy-PTIO (Fig. 5 and 6, respectively). However, maximal inhibition of NE release in PVN and AMYG by either agent was only approximately 70%, indicating that NTS NO mediates a large fraction, but not all, of the NE release triggered by nicotine-induced glutamate release and acting through NTS NMDA receptors (dosages greater than 30 nmol of L- NAME or 0.2 nmol of carboxy-PTIO were not evaluated since a small increase in locomotion, grooming and nodding was evident at these concentrations). These observations, in conjunction with the aforementioned neuroanatomical studies, support the concept that nicotine indirectly activates NTS NE neurons projecting to PVN and AMYG by directly stimulating NTS glutamate release, which in turn activates nNOS in neurons containing NMDA receptors. These results also are consistent with an *in vivo* study demonstrating that nicotine-stimulated NO production in rat hippocampus depends on glutamate release via presynaptic nicotinic cholinergic receptors and the subsequent activation of NMDA receptors (Fedele et al., 1998).

Glutamatergic visceral afferents are the principal excitatory innervation of the NTS and NMDA receptor activation induces excitatory post-synaptic currents on second-order

NTS neurons (Bonham and Chen, 2002). These second order neurons may contain a wide array of neurotransmitters and neuropeptides, with 21 phenotypically-identified perikarya throughout the NTS (Palkovits, 1984; Leslie, 1985). A relatively large number of NTS neurons contain somatostatin, bombesin, TRH, substance P and Met-enkephalin, while fewer cells express Leu-enkephalin, α-endorphin, dynorphin, ACTH, vasopressin, and CCK. VIP is contained within interneurons, and recent studies indicate that NMDA receptors mediate the release of neurokinins (e.g., Substance P) from some interneurons (Maley, 1996; Colin et al., 2002). Substance P appears to attenuate vagally mediated cardiac baroreflexes through the activation of neurokinin receptors (i.e., NK1) on NTS GABA interneurons (Pickering et al., 2003). However, these mechanisms spare sympathetic cardiac baroreflexes, suggesting that NE efferents projecting to PVN and AMYG also would be unaffected. Since previous experiments from our laboratory have shown that a majority of NTS neurons expressing c-Fos in response to i.v. nicotine, were not catecholaminergic (Valentine et al., 1996), the phenotype of NTS neurons activated by nicotine remains largely unidentified. It is probable that many of these neurons express NMDA receptors and respond to the glutamate released by nicotine, in turn secreting an unknown neurotransmitter(s) that would account for the NMDA receptor-dependent, NO-independent fraction (i.e. 20%) of NE released in PVN and AMYG by systemic nicotine. Part of this NO-independent fraction might also be attributed to the direct effect of NTS glutamate on NE neurons, since some of these neurons express NMDA receptor protein (unpublished observations by SG Matta, Ph.D. and BM Sharp).

In present study, 70% of the NE released in the ipsilateral PVN and AMYG by systemic nicotine was inhibited by a unilateral NTS microinjection of AP-5, and bilateral AP-5 microinjections increased this inhibition to approximately 90%. These results are pharmacological corroboration of neuroanatomical evidence demonstrating that a majority of NTS noradrenergic fibers ascend on the same side and innervate the ipsilateral PVN (Palkovits et al., 1999). The remaining 10% of NE release, unaffected by a bilateral NTS AP-5 microinjection, is potentially due to noradrenergic afferents that do not originate from NTS but are responsive to systemic nicotine. In this regard, both PVN and AMYG receive noradrenergic projections from other brain regions, primarily locus coeruleus (LC), and these LC NE projections are responsive to nicotine (Matta et al., 1993; Matta et al., 1993; Fu et al., 1998). Based on the efficacy of conotoxins (i.e. MII and AuIB) microinjected in LC, which specifically inhibit nAChRs containing $\alpha3$ or $\alpha6$ subunits, we also have reported that nicotine, instilled locally activates noradrenergic neurons in LC (Fu et al., 1999). However, the present study demonstrates that a moderate dose of systemic nicotine (i.e., 0.065 mg/kg over 44 sec) predominantly affects NE secretion in the PVN and AMYG through direct effects on neurotransmission within the NTS. Also, studies of cFos expression indicate that LC is less sensitive to systemic nicotine than the NTS (Matta et al., 1993; Matta et al., 1993; Valentine et al., 1996). Therefore, the NTS appears to be the primary site whereby glutamatergic afferents and NO are essential mediators of the NE response to systemic nicotine.

In summary, both the PVN and AMYG are primary regions involved in brain responses to external stressors, including those that affect the hypothalamic-pituitary-adrenal axis,

to emotional memory, and to cue- or stressor-dependent reinstatement of drug seeking behavior (See, 2002). Noradrenergic afferents to these two regions are essential for their function and chronic nicotine self-administration has been shown to enhance this NE release. The present studies demonstrate that both local and systemic nicotine, acting directly through the NTS, induce NE secretion in the PVN and AMYG. However, the noradrenergic neurons themselves are not the direct targets of nicotine's actions, based on the ability of intra-NTS AP-5 to completely block nicotine-stimulated NE release. It is the nicotine-induced glutamate release in NTS that, in turn, activates NMDA receptors, most likely inducing local NO production, which mediates most of the noradrenergic response.

References

Arroyo M, Markou A, Robbins TW and Everitt BJ (1998) Acquisition, maintenance and reinstatement of intravenous cocaine self-administration under a second-order schedule of reinforcement in rats: effects of conditioned cues and continuous access to cocaine. *Psychopharmacology (Berl)* 140:331-344.

Ashworth-Preece M, Jarrott B and Lawrence AJ (1998) Nicotinic acetylcholine receptors in the rat and primate nucleus tractus solitarius and on rat and human inferior vagal (nodose) ganglia: evidence from in vivo microdialysis and [125I]alpha-bungarotoxin autoradiography. *Neuroscience* 83:1113-1122.

Ashworth-Preece MA, Jarrott B and Lawrence AJ (1998) Nicotinic acetylcholine receptor mediated modulation of evoked excitatory amino acid release in the nucleus tractus solitarius of the rat: evidence from in vivo microdialysis. *Brain Res* 806:287-291.

Bonham AC and Chen CY (2002) Glutamatergic neural transmission in the nucleus tractus solitarius: N-methyl-D-aspartate receptors. *Clin Exp Pharmacol Physiol* 29:497-502.

Boscan P, Pickering AE and Paton JF (2002) The nucleus of the solitary tract: an integrating station for nociceptive and cardiorespiratory afferents. *Exp Physiol* 87:259-266.

Buller KM (2003) Neuroimmune stress responses: reciprocal connections between the hypothalamus and the brainstem. *Stress* 6:11-17.

Cahill L, Haier RJ, Fallon J, Alkire MT, Tang C, Keator D, Wu J and McGaugh JL

(1996) Amygdala activity at encoding correlated with long-term, free recall of emotional information. *Proc Natl Acad Sci U S A* 93:8016-8021.

Colin I, Blondeau C and Baude A (2002) Neurokinin release in the rat nucleus of the solitary tract via NMDA and AMPA receptors. *Neuroscience* 115:1023-1033.

Dani JA and De Biasi M (2001) Cellular mechanisms of nicotine addiction. *Pharmacol Biochem Behav* 70:439-446.

Fedele E, Varnier G, Ansaldo MA and Raiteri M (1998) Nicotine administration stimulates the in vivo N-methyl-D-aspartate receptor/nitric oxide/cyclic GMP pathway in rat hippocampus through glutamate release. *Br J Pharmacol* 125:1042-1048.

Ferreira M, Jr., Sahibzada N, Shi M, Panico W, Niedringhaus M, Wasserman A, Kellar KJ, Verbalis J and Gillis RA (2002) CNS site of action and brainstem circuitry responsible for the intravenous effects of nicotine on gastric tone. *J Neurosci* 22:2764-2779.

Ferry B, Roozendaal B and McGaugh JL (1999) Role of norepinephrine in mediating stress hormone regulation of long-term memory storage: a critical involvement of the amygdala. *Biol Psychiatry* 46:1140-1152.

Fu Y, Matta SG, James TJ and Sharp BM (1998) Nicotine-induced norepinephrine release in the rat amygdala and hippocampus is mediated through brainstem nicotinic cholinergic receptors. *J Pharmacol Exp Ther* 284:1188-1196.

Fu Y, Matta SG, Kane VB and Sharp BM (2003) Norepinephrine release in amygdala of rats during chronic nicotine self-administration: an in vivo microdialysis study. *Neuropharmacology* 45:514-523.

Fu Y, Matta SG, McIntosh JM and Sharp BM (1999) Inhibition of nicotine-induced hippocampal norepinephrine release in rats by alpha-conotoxins MII and AuIB microinjected into the locus coeruleus. *Neurosci Lett* 266:113-116.

Fu Y, Matta SG, Valentine JD and Sharp BM (1997) Adrenocorticotropin response and nicotine-induced norepinephrine secretion in the rat paraventricular nucleus are mediated through brainstem receptors. *Endocrinology* 138:1935-1943.

Galvez R, Mesches MH and McGaugh JL (1996) Norepinephrine release in the amygdala in response to footshock stimulation. *Neurobiol Learn Mem* 66:253-257.

Hatfield T, Spanis C and McGaugh JL (1999) Response of amygdalar norepinephrine to footshock and GABAergic drugs using in vivo microdialysis and HPLC. *Brain Res* 835:340-345.

Lambe EK, Picciotto MR and Aghajanian GK (2003) Nicotine induces glutamate release from thalamocortical terminals in prefrontal cortex. *Neuropsychopharmacology* 28:216-225.

Leslie RA (1985) Neuroactive substances in the dorsal vagal complex of the medulla oblongata: nucleus of the tractus solitarius, area postrema, and dorsal motor nucleus of the vagus. *Neurochemistry International* 7:191-211.

Lin LH and Talman WT (2000) N-methyl-D-aspartate receptors on neurons that synthesize nitric oxide in rat nucleus tractus solitarii. *neuroscience* 100:581-588.

Lonart G, Wang J and Johnson KM (1992) Nitric oxide induces neurotransmitter release from hippocampal slices. *Eur J Pharmacol* 220:271-272.

Maley BE (1996) Immunohistochemical localization of neuropeptides and neurotransmitters in the nucleus solitarius. *Chem Senses* 21:367-376.

Matsuo I, Hirooka Y, Hironaga K, Eshima K, Shigematsu H, Shihara M, Sakai K and
Takeshita A (2001) Glutamate release via NO production evoked by NMDA in
the NTS enhances hypotension and bradycardia in vivo. *Am J Physiol Regul
Integr Comp Physiol* 280:R1285-1291.

Matta SG, Foster CA and Sharp BM (1993) Nicotine stimulates the expression of cFos
protein in the parvocellular paraventricular nucleus and brainstem
catecholaminergic regions. *Endocrinology* 132:2149-2156.

Matta SG, Foster CA and Sharp BM (1993) Selective administration of nicotine into
catecholaminergic regions of rat brainstem stimulates adrenocorticotropin
secretion. *Endocrinology* 133:2935-2942.

Matta SG, Fu Y, Valentine JD and Sharp BM (1998) Response of the hypothalamo-
pituitary-adrenal axis to nicotine. *Psychoneuroendocrinology* 23:103-113.

Matta SG, Singh J and Sharp BM (1990) Catecholamines mediate nicotine-induced
adrenocorticotropin secretion via alpha-adrenergic receptors. *Endocrinology*
127:1646-1655.

McGaugh JL and Izquierdo I (2000) The contribution of pharmacology to research on the
mechanisms of memory formation. *Trends Pharmacol Sci* 21:208-210.

Oh S (1995) The generation of nitric oxide and its roles in neurotransmission and
neurotoxicity. *Keio J Med* 44:53-61.

Ohno M, Yamamoto T and Watanabe S (1993) Amygdaloid NMDA and muscarinic
receptors involved in working memory performance of rats. *Physiol Behav*
54:993-997.

Palkovits M (1984) Distribution of neuropeptides in the central nervous system: a review

of biochemical mapping studies. *Prog Neurobiol* 23:151-189.

Palkovits M, Baffi JS and Pacak K (1999) The role of ascending neuronal pathways in stress-induced release of noradrenaline in the hypothalamic paraventricular nucleus of rats. *J Neuroendocrinol* 11:529-539.

Paxinos G and Watson C (1986) *the Rat Brain in Stereotaxic Coordinates*. Academic Press, New York.

Pickering AE, Boscan P and Paton JF (2003) Nociception attenuates parasympathetic but not sympathetic baroreflex via NK1 receptors in the rat nucleus tractus solitarii. *J Physiol* 551:589-599.

Reid MS, Fox L, Ho LB and Berger SP (2000) Nicotine stimulation of extracellular glutamate levels in the nucleus accumbens: neuropharmacological characterization. *Synapse* 35:129-136.

See RE (2002) Neural substrates of conditioned-cued relapse to drug-seeking behavior. *Pharmacol Biochem Behav* 71:517-529.

Valentine JD, Matta SG and Sharp BM (1996) Nicotine-induced cFos expression in the hypothalamic paraventricular nucleus is dependent on brainstem effects: correlations with cFos in catecholaminergic and noncatecholaminergic neurons in the nucleus tractus solitarius. *Endocrinology* 137:622-630.

Williams CL, Men D and Clayton EC (2000) The effects of noradrenergic activation of the nucleus tractus solitarius on memory and in potentiating norepinephrine release in the amygdala. *Behav Neurosci* 114:1131-1144.

Zhang J and Mifflin SW (1998) Differential roles for NMDA and non-NMDA receptor subtypes in baroreceptor afferent integration in the nucleus of the solitary tract of

the rat. *J Physiol* 511 (Pt 3):733-745.

Footnotes:

This research was supported by DA-03977 (B.M.S.) from NIDA.

Legends for Figures

Fig. 1. Diagrammatic representation of dialysis probe placement in the rat PVN or AMYG and microinjection sites into NTS-A2. Histological analysis of cryostat rat brain coronal sections was used to verify the position of the membrane segment of each microdialysis probe and of the microinjection cannulae in all animals; these are indicated by *line segments.* PVN is 2.1 mm, AMYG is 2.8 mm and NTS is 13.6 mm posterior to bregma in diagrams adapted from Paxinos and Watson (Paxinos and Watson, 1986).

Fig. 2. The NMDA receptor antagonist, AP-5, dose-dependently blocked the NE release in PVN (A) and AMYG (B) elicited by a microinjection of nicotine into NTS. AP-5 0.75 µg or 1.5 µg (or KRB) were unilaterally microinjected into NTS-A2 5 min prior to a unilateral intra-NTS microinjection of KRB or 1.2 µg of nicotine (Nic); peak NE levels were expressed as a percentage of the average of 3 baseline levels prior to drug administration. Basal PVN NE levels for KRB/Nic, AP-5 (.75)/Nic, AP-5 (1.5)/Nic, AP-5 (1.5)/KRB, and KRB/KRB treatment groups were 4.1 ± 0.5 (nM), 3.8 ± 0.5, 3.7 ± 0.4, 4.2 ± 0.6 and 4.3 ± 0.6, respectively. Basal AMYG NE levels in KRB/Nic, AP-5 (.75)/Nic, AP-5 (1.5)/Nic, AP-5 (1.5)/KRB, and KRB/KRB treatment groups were 1.4 ± 0.2 (nM), 1.3 ± 0.1, 1.5 ± 0.3, 1.4 ± 0.2 and 1.3 ± 0.2, respectively. Panel A: intra-NTS AP-5 0.75 µg and 1.5 µg significantly inhibited the effect of a subsequent intra-NTS microinjection of nicotine on stimulating NE release in the PVN by 63 ± 2.1 % and 98.8 ± 1.1 %, respectively. Panel B: a similar dose-dependent inhibition by intra-NTS AP-5 on nicotine-stimulated NE release in AMYG was observed. Pre-treatment with intra-NTS

AP-5 (0.75 µg and 1.5 µg) significantly inhibited the effect of intra-NTS nicotine on NE release in the AMYG by 54.0 ± 1.6 % and 104.8 ± 2.0 %, respectively. $*p < 0.05$; $**p < 0.01$ compared with KRB/KRB group at the same time point; $^{\#\#}p < 0.01$ compared with KRB/Nic group at the same time point (n = 4-6 rats per cohort).

Fig. 3. The AMPA receptor antagonist, CNQX, was ineffective in blocking intra-NTS nicotine-induced NE release in PVN (A) and AMYG (B). CNQX 1.0 µg or 2.0 µg (or KRB) were microinjected unilaterally into NTS 5 min prior to a microinjection of 1.2 µg nicotine or KRB. Basal NE levels were 4.1 ± 0.6 nM in PVN and 1.5 ± 0.2 nM in AMYG. (A) In PVN, neither dose of CNQX reduced nicotine-stimulated levels of NE. Similarly, intra-NTS CNQX was ineffective in blocking nicotine-stimulated NE release in AMYG (B). $*p < 0.05$; $^{\#}p < 0.05$; $^{\$}p < 0.05$ compared with KRB/Nic, CNQX (1.0)/Nic and CNQX (2.0)/Nic respectively (n = 4-5 rats per cohort).

Fig. 4. Intra-NTS micoinjection of AP-5 also blocks NE release in PVN (A, C) and AMYG (B, D) in response to systemic nicotine. AP-5 1.5 µg/100 nl/30 sec was microinjected unilaterally (A-B) or bilaterally (C and D) into NTS 5 min prior to nicotine 0.09 mg/kg/60 sec (A and B) or nicotine 0.065 mg/kg/44 sec (C and D). A unilateral (Uni) microinjection of intra-NTS AP-5 1.5 µg significantly reduced NE release by 61.3 ± 10.2 % in PVN (A) and 56.9 ± 8.5 % in AMYG (B) in response to the higher dose of nicotine (0.09 mg/kg). $*p < 0.05$ or $**p < 0.01$ compared to Uni AP-5 (1.5)/Sal controls; $^{\#}p < 0.05$ compared with KRB/Nic (.09) treatment group (n = 4-5 rats per cohort). These studies were extended to compare the efficacy of a unilateral (Uni) vs. bilateral (Bi) AP-5 intra-NTS microinjection on blocking the NE response to a lower (Nic 0,065 mg.kg) dose

of systemic nicotine. In PVN (C), unilateral AP-5 blocked NE release by 70.8 ± 4.0 % (compared to 63.0 ± 3.0 % in AMYG), and a bilateral microinjection further reduced inhibition to approximately 89.5 ± 4.4 % (compared to 83.5 ± 4.0 % in AMYG). *$p <$ 0.05 or **$p < 0.01$ compared with Bi AP-5 (1.5)/Sal treatment group; [#] $p < 0.05$ compared with KRB/Nic (.065) group; [$] $p < 0.05$ compared with Bi AP-5 (1.5)/Nic (.065) group. Basal NE levels were 4.2 ± 0.5 nM in PVN (A, C) and 1.4 ± 0.2 nM in AMYG (B, D). (n = 4-6 rats per cohort).

Fig. 5. Inhibition of NOS in the NTS with L-NAME partially reduced NE release in PVN (A) and AMYG (B) in response to systemic nicotine. 10 nmol or 30 nmol of L-NAME was bilaterally microinjected into NTS 5 min prior to i.v. nicotine (0.065 mg/kg/44 sec). The average basal NE level in PVN (A) was 3.9 ± 0.5 (nM) and 1.3 ± 0.2 (nM) in AMYG (B). In PVN, 10 nmol L-NAME in NTS blocked nicotine-induced NE release by 52.3 ± 3.5 % (compared to 42.6 ± 3.5 % in AMYG) and by 71.1 ± 3.6 % (compared to 63.7 ± 3.5 % in AMYG) when 30 nmol was microinjected. * $p < 0.05$ or **$p < 0.01$ compared with L-NAME (30)/Sal control group; [#]p < 0.05 compared with KRB/Nic (.065) treatment group; [$]$p < 0.05$ compared with L-NAME (30)/Nic (.065) group (n = 4-5 rats per cohort).

Fig. 6. Intr-aNTS microinjection of the NO scavenger, carboxy-PTIO reducedNE release in PVN (A) and AMYG (B) in response to systemic nicotine. 0.1 nmol or 0.2 nmol of carboxy-PTIO was bilaterally microinjected into NTS 5 min prior to i.v. nicotine (0.065 mg/kg/44 sec). The average basal NE level in PVN (A) was 4.0 ± 0.5 nM and 1.4 ± 0.2
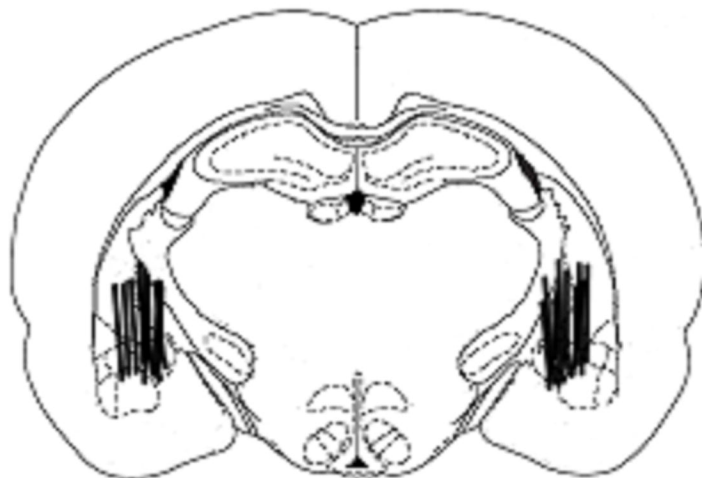
nM in AMYG (B). Removal of diffusible NO from NTS with 0.1 nmol carboxy-PTIO

reduced nicotine-stimulated NE release by 41. 7 ± 4.4 % in PVN (compared to 40.0 ± 3.8

% in AMYG), whereas 0.2 nmol produced a 67.5 ± 4.1 % reduction (compared to 62.4 ±

3.7 % in AMYG). *$p < 0.05$ or **$p < 0.01$ compared with PTIO (0.2)/Sal controls; [#]$p <$

0.05 compared with KRB/Nic treatment group; [$]$p < 0.05$ compared with carboxy-PTIO

(0.2)/Nic group (n = 4-5 rats per cohort).
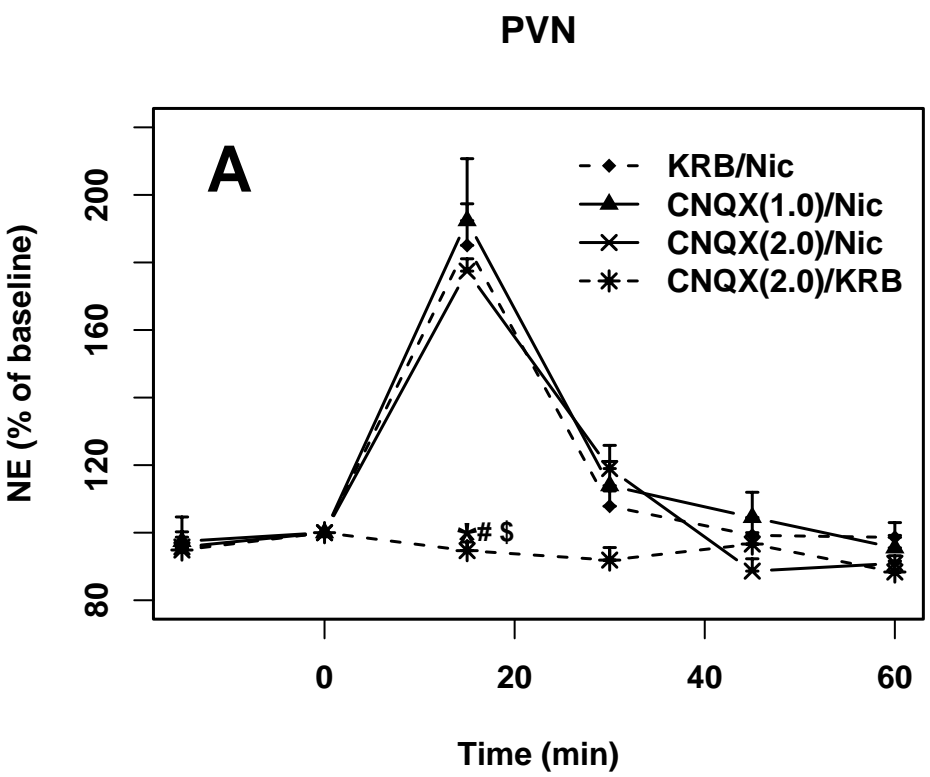
Figure 1

PVN

AMYG

NTS

Figure 2

PVN

AMYG

Figure 3

Figure 4

Figure 5

PVN

AMYG

Figure 6

PVN

AMYG