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# Nicotine-Induced Norepinephrine Release in the Rat Amygdala and Hippocampus is Mediated through Brainstem Nicotinic Cholinergic Receptors[1]

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## ABSTRACT

Previous studies have shown that nicotine stimulates norepinephrine (NE) release in the rat hypothalamic paraventricular nucleus, which in turn activates the hypothalamo-pituitary-adrenal axis. In the present study, nicotine induced NE release in the amygdala (AMYG) and the hippocampus (HP) of the same rat *in vivo*. Nicotine (0.065–0.135 mg/kg i.v. at a rate of 0.09 mg/kg/60 sec) dose-dependently increased NE release at both sites with similar potencies. To determine whether the site of action of nicotine is in the brainstem, which contains the noradrenergic cell bodies projecting to AMYG and HP, nicotinic cholinergic receptor (NAchR) antagonists were injected into the cerebral aqueduct before i.v. nicotine. Use of the following antagonists enabled partial characterization of the NAchRs mediating NE secretion: mecamylamine (Mec), dihydro-$\beta$-erythroidine (DH$\beta$E), methyllycaconitine (MLA) and $\alpha$-bungarotoxin ($\alpha$-BTX). Mec inhibited 80% of NE release in AMYG and 87% in HP ($IC_{50}$ = 6 nmol for both regions). DH$\beta$E blocked 62% of NE release in AMYG ($IC_{50}$ = 8 nmol) and 63% in HP ($IC_{50}$ = 15 nmol). Similar to DH$\beta$E, MLA inhibited 60% of NE release in AMYG and 66% in HP ($IC_{50}$ = 5 nmol for both regions). In contrast, $\alpha$-BTX had no effect on NE release in either region. These results indicate that brainstem NAchRs accessible from the fourth ventricle mediate nicotine-stimulated NE secretion in AMYG and HP. Taken together with prior investigations showing the brainstem expression of mRNAs encoding NAchR subtypes and the selectivity of antagonists for NAchR subtypes, the present studies suggest that brainstem *alpha*-3 subunits may be involved.



Nicotine is a psychoactive component in cigarette smoke that affects many physiological functions of the CNS. By stimulating brainstem noradrenergic and peptidergic pathways, nicotine activates the HPA axis, which leads to the secretion of stress-responsive hormones (Sharp and Beyer, 1986; Matta *et al.*, 1990; Valentine, *et al.*, 1996; Fu *et al.*, 1997). Memory-enhancing effects of nicotine also have been reported in both human and animal studies (Warburton, 1990; McGehee and Role, 1996). Indeed, a loss of NAchRs was found in Alzheimer's disease (Schroder *et al.*, 1991), and the administration of intravenous nicotine to these patients appeared to improve memory transiently (Newhouse *et al.*, 1988, 1990).

The central noradrenergic system is involved in stress-related responses and memory function (Bremner *et al.*, 1996). Most CNS noradrenergic cell bodies are located in the LC, although a large group also are found in the NTS-A2 and the ventromedullary A1 region (Holets, 1990; Aston-Jones *et al.*, 1995). These central noradrenergic neurons innervate brain regions such as the AMYG, HP, hypothalamus and frontal cortex, which also are anatomical substrates for stress responses and/or memory function (Holets, 1990; Bremner *et al.*, 1996). Systemic nicotine stimulates NE release *in vivo* in the rat hypothalamic PVN (Sharp *et al.*, 1993; Matta *et al.*, 1995; Fu *et al.*, 1997), HP (Brazell *et al.*, 1991; Mitchell, 1993) and the cerebral cortex (Summers and Giacobini, 1995).

The amygdaloid complex is one of the limbic system structures which facilitate HPA axis responses (Feldman and Weidenfeld, 1996) and govern a wide array of autonomic functions. In addition, AMYG plays an essential role in emotional memory (Cahill *et al.*, 1996; LeDoux, 1992; Izquierdo and Medina, 1995; Maren, 1996), in working memory performance (Ohno *et al.*, 1993) and in the regulation of the storage of memory by other brain regions (McGaugh *et al.*, 1990; Galvez *et al.*, 1996). Evidence indicates that the release of NE

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**ABBREVIATIONS:** AMYG, amygdala; $\alpha$-BTX, $\alpha$-bungarotoxin; DH$\beta$E, dihydro-$\beta$-erythroidine; HP, hippocampus; HPA, hypothalamo-pituitary-adrenal; HPLC, high-performance liquid chromatography; KRB, Kreb's Ringer Buffer; LC, locus coeruleus; Mec, mecamylamine; MLA, methyllycaconitine; NAchRs, nicotinic cholinergic receptors; NE, norepinephrine; NTS, nucleus tractus solitarius; PVN, hypothalamic paraventricular nucleus; CNS, central nervous system; CSF, cerebrospinal fluid; EDTA, ethylenediaminetetraacetic acid.

is involved in both AMYG-mediated HPA responses (Feldman and Weidenfeld, 1996) and memory function (Introini-Collison *et al.*, 1996; McGaugh *et al.*, 1988; Cahill and McGaugh, 1996). However, no studies have examined the effect of nicotine on NE release in the AMYG.

The HP is another limbic region known for its role in memory development (Lee *et al.*, 1993) and HPA responses (Feldman *et al.*, 1995). Direct connections between the HP and AMYG may be important in the limbic memory system (Saunders *et al.*, 1988; Izquierdo and Medina, 1995). In addition, studies have shown that NE is involved in hippocampal functions that enhance memories of inhibitory avoidance and spatial habituation (Izquierdo *et al.*, 1992; Izquierdo and Medina, 1995). It has been reported that systemically administered nicotine stimulates NE release in the HP and is sensitive to antagonist blockade (Mitchell, 1993). In that study, only Mec, an NAchR antagonist with limited specificity (Olney *et al.*, 1978; Clarke *et al.*, 1994), was tested. Thus, the subtype(s) of the NAchRs mediating systemic nicotine-induced NE release in the HP is not known.

Neuronal NAchRs are pentameric receptors consisting of *alpha* (agonist binding) and *beta* subunits. To date, eight different *alpha* subunits (*alpha* 2 to 9) as well as four *beta* subunits (*beta* 2 to 5) have been identified (Lukas, 1995; McGehee and Role, 1995; Vidal, 1996). Distinct receptor subtypes, consisting of various combinations of *alpha* and *beta* subunits, have been shown to co-exist in many brain regions, including the AMYG and HP (Wada *et al.*, 1989; Flores *et al.*, 1992; Rubboli *et al.*, 1994). Several nicotinic antagonists have been described which are suitable for *in vivo* pharmacological investigations of these subtypes. Mec is, perhaps, the most commonly used antagonist. It is an ion channel blocker (Varanda *et al.*, 1985) and is most effective at *alpha*-3 *beta*-4 receptors (Cachelin and Rust, 1995; Alkondon and Albuquerque, 1993). DH$\beta$E, a competitive antagonist, is most effective at *alpha*-4 *beta*-2 receptors (Luetje *et al.*, 1990; Alkondon and Albuquerque, 1993). DH$\beta$E inhibits nicotine-elicited excitatory amino acid release in spinal cord (Khan *et al.*, 1996) and reduces the number of infusions of self-administered nicotine (Corrigall *et al.*, 1994). MLA, a toxin isolated from *Delphinium* sp., is another competitive NAchR blocker. It potently blocks $\alpha$-BTX-sensitive *alpha*-7-containing NAchR at nanomolar concentrations (Alkondon *et al.*, 1992). In contrast, micromolar concentrations of MLA are required to inhibit the response to $\alpha$-BTX-insensitive (non- *alpha*-7) receptors (Alkondon and Albuquerque, 1993). Therefore, multiple antagonists can be used to obtain pharmacological evidence for the subtype(s) of NAchR(s) that is involved in nicotine-stimulated NE release in specific regions of the brain.

In the present studies, NE release in AMYG and HP was detected concurrently in the same rat by *in vivo* microdialysis. Initial experiments were performed to establish dose-response relationships for NE release in both the AMYG and HP in response to i.v. infusions of nicotine. Then, experiments were performed to determine whether nicotine acts through receptors located in the brainstem, which harbors the noradrenergic cell bodies that project to the AMYG and HP. The NAchR subtypes mediating NE secretion in the AMYG and HP were characterized pharmacologically by determining the relative efficacies and potencies of the following NAchR antagonists: Mec, DH$\beta$E, MLA and $\alpha$-BTX.

## Materials and Methods

**Materials.** Nicotine sulfate (Pfaltz and Bauer, Inc., Waterbury, CT; all dosages are given as milligrams per kilogram of the free base) was used for i.v. injection. Norepinephrine hydrochloride, mecamylamine hydrochloride, dihydro-$\beta$-erythroidine hydrobromide, methyllycaconitine citrate, $\alpha$-bungarotoxin and nomifensine maleate were purchased from RBI (Natick, MA). Sodium dihydrogen phosphate monohydrate (EM Science, Gibbstown, NJ), 1-octanesulfonic acid sodium salt (J.T. Baker, Phillipsburg, NJ), triethylamine (Aldrich, Milwaukee, WI), EDTA (Fisher Scientific, Minneapolis, MN), acetonitrile and phosphoric acid (EM Science, HPLC grade) were used to prepare the mobile phase. The alert-rat microdialysis systems and CMA 110 liquid switches were obtained from CMA/Microdialysis (Acton, MA). For constructing dialysis probes, cellulose fiber tubing was obtained from Spectrum (Laguna Hills, CA), and silica tubing (outside diameter, 148 $\mu$m; internal diameter, 73 $\mu$m) was from Polymicron Technologies Inc. (Phoenix, AZ).

**Animals.** Adult male Holtzman rats (250–350 g, HSD, Madison, WI) were given access to standard rat chow and water *ad libitum*. They were housed individually on a 12-h reversed light cycle (lights off at 9 A.M., on at 9 P.M.) for 14 days before the microdialysis experiments. After the rats had been housed under this reversed light/dark cycle for 7 days, they were anesthetized with xylazine ketamine (5.35 mg/kg b.wt. i.m.; Parke-Davis, Morris Plains, NJ) and chronic guide cannulae were implanted stereotaxically into AMYG (right side) and HP (left side) in the same rat, according to the coordinates of Paxinos and Watson (1986). Some cohorts also were implanted with guide cannulae into the cerebral aqueduct. The coordinates for AMYG were AP, −2.5 mm; DV, −7.2 mm; ML, 5.0 mm from bregma with a flat skull; HP coordinates were AP, −3.0 mm; DV, −2.6 mm; ML, 1.4 mm, from bregma with a flat skull; cerebral aqueduct coordinates were AP, +0.8 mm, DV, +5.2 mm, ML, 0.0 mm, relative to lambda and the interaural line with flat skull.

Five days later, rats were equipped with jugular cannulae under Innovar Vet anesthesia (droperidol, 3.75 mg/kg, plus fentanyl, 0.08 mg/kg i.m.; Far-Vet, St Paul, MN) and allowed to recover for another 2 days. 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 Minneapolis Medical Research Foundation.

***In vivo* microdialysis.** A small, concentric probe (MW cutoff, 13,000 daltons; outside diameter, 235 $\mu$m; 1-mm length for AMYG and 2-mm length for HP; constructed in our laboratory, Fu *et al.*, 1997) was used in this study. The recovery rate of individual probes was determined by *in vitro* dialysis of single probes for 60 min at 22°C in a solution of 200 pg NE/16 $\mu$l. The probes were perfused at 1 $\mu$l/min with standard perfusate (KRB; see below), and three 20-min samples were obtained. The average recovery rate was 4.2% ± 0.6 (mean ± S.D., $n$ = 15 probes) for the AMYG 1-mm probe and 7.5% ± 0.7 ($n$ = 15) for the HP 2-mm probe.

The procedures were carried out as described previously (Fu *et al.*, 1997). On the day of microdialysis, rats were moved into the alert-rat microdialysis chambers in an isolated dark room lit with a red safe-light, and all connections were made quickly to minimize stress to the animal. The probe was perfused at 1 $\mu$l/min with a solution of KRB (147 mM NaCl, 4.0 mM KCl and 3.4 mM $CaCl_2$ in polished water; 0.2 $\mu$m filter sterilized and degassed) containing 5 $\mu$M nomifensine (NE uptaker blocker, Schacht *et al.*, 1982). Two hours after insertion of the probe, three consecutive samples were collected to measure basal levels before drug administration. Samples were each collected for 20 min into vials containing 1 $\mu$l of 5% perchloric acid to prevent the degradation of NE.

At the end of the experiments, the position of the probe was verified by histological examination (see fig. 1); only data obtained from animals with probes identified in the correct location of the AMYG and HP were used for analysis. Placement of cannulae in the cerebral aqueduct was assessed by microinjection of 1 $\mu$l trypan blue;

data were analyzed only from rats with blue in the fourth ventricle and none in the surrounding tissue.

**HPLC-electrochemical analysis.** Dialysis samples (16 μl) were immediately injected by a CMA 200 refrigerated autosampler onto a 150 × 3 mm ODS C18 column (ESA Inc., Chelmsford, MA) perfused by BAS 200A HPLC pumps at 0.5 ml/min with a mobile phase containing 80 mM sodium dihydrogen phosphate monohydrate, 2.0 mM 1-octanesulfonic acid sodium salt, 100 μl/l triethylamine, 5 nM EDTA and 10% acetonitrile, pH 3.0. Samples were analyzed by an ESA Coulochem II 5200A electrochemical detector with an ESA 5041 high-sensitivity microbore analytical cell and an ESA 5020 guard cell (ESA). Electrochemical detection was performed at 220 mV and 1.0 nA with the guard cell at 350 mV. The limit of detection for NE was 0.5 pg.

**Experimental protocols.** A preliminary experiment was performed to determine the stability of both the basal NE levels and the responses to nicotine with repeated testing of each rat with a single probe. For each day's experiment, three consecutive preinfusion (basal) microdialysis samples were each collected for 20 min, and then nicotine was infused i.v. at 0.135 mg/kg for 90 sec, whereas dialysates were collected continuously at 20-min intervals for 40 min. This procedure was repeated on d3 and d5 in the same cohort of rats. The results showed that basal levels of NE in AMYG were reduced significantly on d3 and d5 compared with d1: 4.4 ± 0.5 pg/16 μl (mean ± S.E.M.) on d1, 2.8 ± 0.4 pg/16 μl on d3 ($P < .05$ compared with d1) and 2.3 ± 0.4 pg/16 μl on d5 ($P < .01$ compared with d1). However, no significant difference was observed between d3 and d5. Similar results were found in HP: 6.6 ± 0.5 pg/16 μl on d1, 4.2 ± 0.7 pg/16 μl on d3 ($P < .05$ compared with d1) and 3.6 ± 0.4 pg/16 μl on d5 ($P < .01$ compared with d1). In response to nicotine, the peak AMYG levels of NE were 7.8 ± 0.9 pg/16 μl on d1, 5.9 ± 0.6 pg/16 μl on d3 and 4.8 ± 0.5 pg/16 μl on d5. In HP, they were 13.8 ± 1.3 pg/16 μl on d1, 9.0 ± 1.1 pg/16 μl on d3 and 7.9 ± 0.8 pg/16 μl on d5. NE levels on d3 and d5 were lower than those detected on d1 in both regions ($P < .05$ compared with d1 for both brain regions). These measurements indicate that basal NE levels and NE responses to nicotine were stable between d3 and d5 in each region. Therefore, in all subsequent experiments, on d1 a probe was inserted for 10 min and removed thereafter without further microdialysis (sham microdialysis). On d3 and d5, probes were reinserted and rats received randomized treatments.

The second experiment was conducted to determine the dose-response relationship for nicotine-induced NE secretion in AMYG and HP. Rats randomly received infusions of saline or one of four doses of nicotine (each delivered at a constant rate of 0.09 mg/kg per 60 sec): 0.045 mg/kg for 30 sec, 0.065 for 44 sec, 0.09 mg/kg for 60 sec or 0.135 mg/kg for 90 sec (Valentine *et al.*, 1996). This dosing regimen was used to avoid aversive behavioral responses that might induce NE release, independently of nicotine. Because the behavioral responses to nicotine 0.135 mg/kg in some rats (brief locomotion and/or brief tremor) indicated that nonspecific, system-wide activation would be elicited by higher doses, the higher doses needed to calculate the exact $ED_{50}$ were unfeasible. Therefore, the $ED_{50}$ doses reported are approximate.

The third experiment was designed to determine whether NAchRs in brainstem regions (*e.g.,* LC, NTS-A2 and A1, the major sites of noradrenergic cell bodies) accessible from the cerebral aqueduct are involved in nicotine-stimulated NE release in AMYG and HP. The cerebral aqueduct was chosen as the injection site because it is immediately rostral to the fourth ventricle and brainstem. Therefore, compounds injected into the aqueduct and carried by the unidirectional flow of CSF will reach downstream brainstem structures accessible from the fourth ventricle, specifically, the LC in the rostral brainstem, as well as the more caudal structures (A1 and NTS-A2). Rats randomly received artificial CSF (300 μg/ml bovine serum albumin in 0.05 M phosphate buffer, pH 7.2), Mec (2, 4, 8 and 16 nmol), DHβE (8.4, 16.8, 28.1 and 84.2 nmol), MLA (0.4, 1.8, 5.4, 10.8 and 32.4 nmol) or α-BTX (1.25 nmol) in 500 nl for 60 sec injected into the cerebral aqueduct; 15 min later rats were infused with saline or 0.09 mg/kg nicotine i.v. for 60 sec. Higher doses of α-BTX were not tested because they elicited agitated behavioral responses in many rats at doses larger than 1.25 nmol (Y. Fu, S. G. Matta and J. D. Valentine, unpublished observations).

To evaluate whether the effects of NAchR antagonists administered into the cerebral aqueduct are localized to the brainstem, two experiments were performed. The first experiment was designed to determine whether a large fraction of the intra-aqueductal dose of an antagonist gaining access to the systemic circulation would effectively inhibit NAchRs at an unspecified site(s). This was assessed by injecting i.v. the $IC_{50}$ dose of Mec (6 nmol/0.1 ml for 60 sec), DHβE (15 nmol/0.1 ml for 60 sec) or CSF 15 min before a nicotine infusion (0.09 mg/kg i.v.). In the second experiment, the hypothetical delivery of NAchR antagonists *via* the CSF circulation to presynaptic NAchR in rostral brain sites was evaluated by administering Mec or DHβE directly into the AMYG and HP through a microdialysis probe. After three 20-min basal samples were collected, perfusates (1 μl/min) containing Mec (80 nmol/20 μl), DHβE (200 nmol/20 μl) or CSF (20 μl) were switched into the inflow catheter (using a CMA 110 liquid switch), and the microdialysis probe was perfused for 20 min. Thereafter, the antagonist solution was replaced by KRB, and 0.09 mg/kg nicotine was infused i.v. Because Mec and DHβE have molecular weights similar to NE, the amount of antagonist that diffused from the probe was estimated from experiments in which the *in vitro* diffusion of NE had been measured. Therefore, the dose of Mec (80 nmol) or DHβE (200 nmol) perfused through the probe was calculated based on 7.5%, the average *in vitro* recovery of NE by HP probes, and on the experimentally determined $IC_{50}$ value of each antagonist.

**Data analysis and statistics.** Chromatographic data were collected and analyzed with the PowerChrom system (AD Instruments, Castle Hill, NSW, Australia) and expressed either as picograms per 16-μl sample or as a percentage of pre-infusion basal NE levels. Basal values were defined in each rat as the average NE levels of the three samples before administration of nicotine, antagonists or vehicle. Data were analyzed by one-way analysis of variance with StatView. Results were considered significant at $P < .05$. The number shown in parentheses ($n$) in the text and graphs is the number of rats within a specific treatment group.

## Results

Representative histological specimens illustrating the placement of concentric dialysis probes in AMYG and HP are shown in figure 1. Each probe was placed to maximally dialyze as much of the specific region as possible, without affecting surrounding structures. Figure 2 shows the HPLC chromatograms obtained from a NE standard (panel A) and representative dialysate samples (panels B–E). The NE peaks are symmetrical, and the retention time of peaks detected in dialysate samples are identical with synthetic NE.

Figure 3, A and B, demonstrates the time course for NE release in the AMYG and HP in response to nicotine infusions during the active (dark) phase of the light cycle. Nicotine stimulated NE release in these two brain regions in a dose-dependent manner. NE concentrations were maximal within the first 20 min after the end of the nicotine infusions and returned to base-line levels immediately thereafter. The maximal responses to nicotine were approximately 2-fold greater than basal NE levels in both brain regions. The potency of nicotine was similar in both regions with approximate $ED_{50}$ values (within the dosage range tested) of 0.073 mg/kg for the AMYG and 0.079 mg/kg for the HP. The specificity of these NE responses to nicotine is underscored by the release of serotonin only at doses greater than 0.09 mg/kg

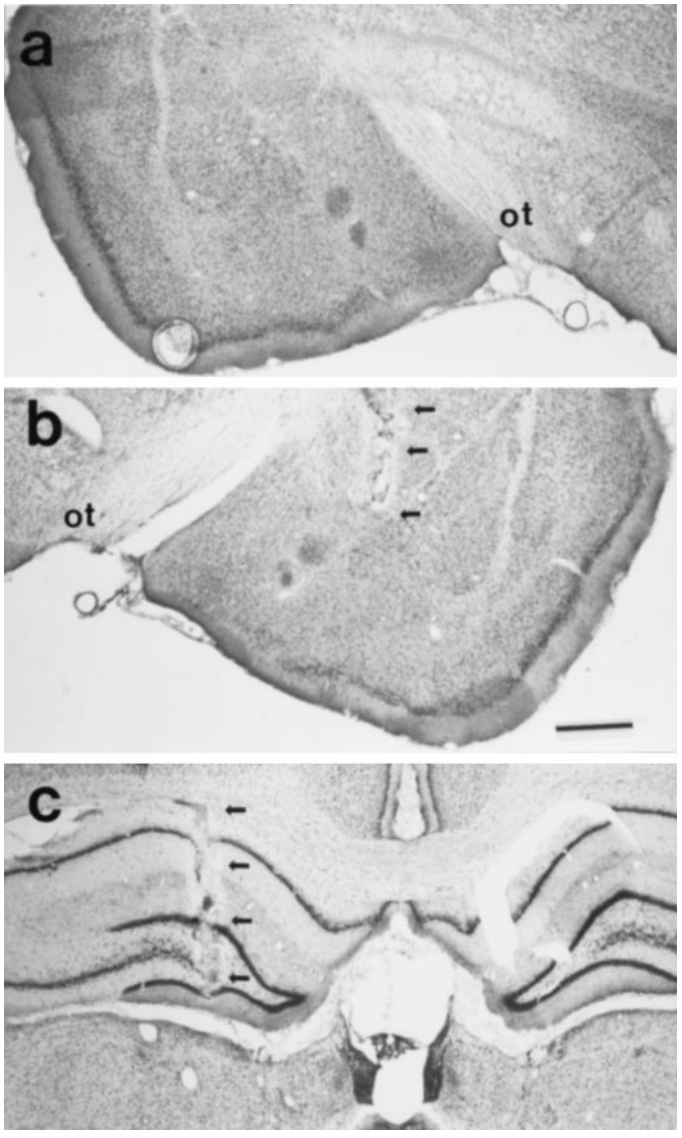


**Fig. 1.** Photomicrographs of probe placement in the AMYG (A, B) and HP (C). Rats were cardiac perfused with 4% paraformaldehyde in 0.05 M phosphate-buffered saline. Brains were removed, cryosectioned at 20 $\mu$m and stained with cresyl violet. Panels A and B show both sides of a coronal section in the same rat: panel A is the intact left AMYG and panel B shows the tissue track of the probe identified by the arrows in the right AMYG. Panel C shows the bilateral HP; the track of the probe is on the left and identified by arrows. Magnification bar, 400 $\mu$m; ot, optic tract.

(data not shown). This dose-dependent release of different neurotransmitters by nicotine indicates specificity, rather than a nonselective secretogogue effect similar to KCl stimulation.

The results presented in figure 4 and table 1 demonstrate that systemic nicotine activated brainstem site(s), leading to NE secretion in the AMYG and HP. Figure 4A shows that nicotine-induced NE release in AMYG was inhibited by Mec, DH$\beta$E and MLA, whereas, by themselves, the antagonists did not affect basal NE levels (data not shown). Doses of Mec equal to or greater than 4 nmol, injected into the cerebral aqueduct, resulted in a dose-dependent blockade of NE release in response to 0.09 mg/kg nicotine. The $IC_{50}$ for Mec blockade of NE release was 6 nmol with 80% maximal inhibition. DH$\beta$E at a dose of 16.8 nmol or greater significantly inhibited NE secretion with an $IC_{50}$ of 8 nmol and maximal

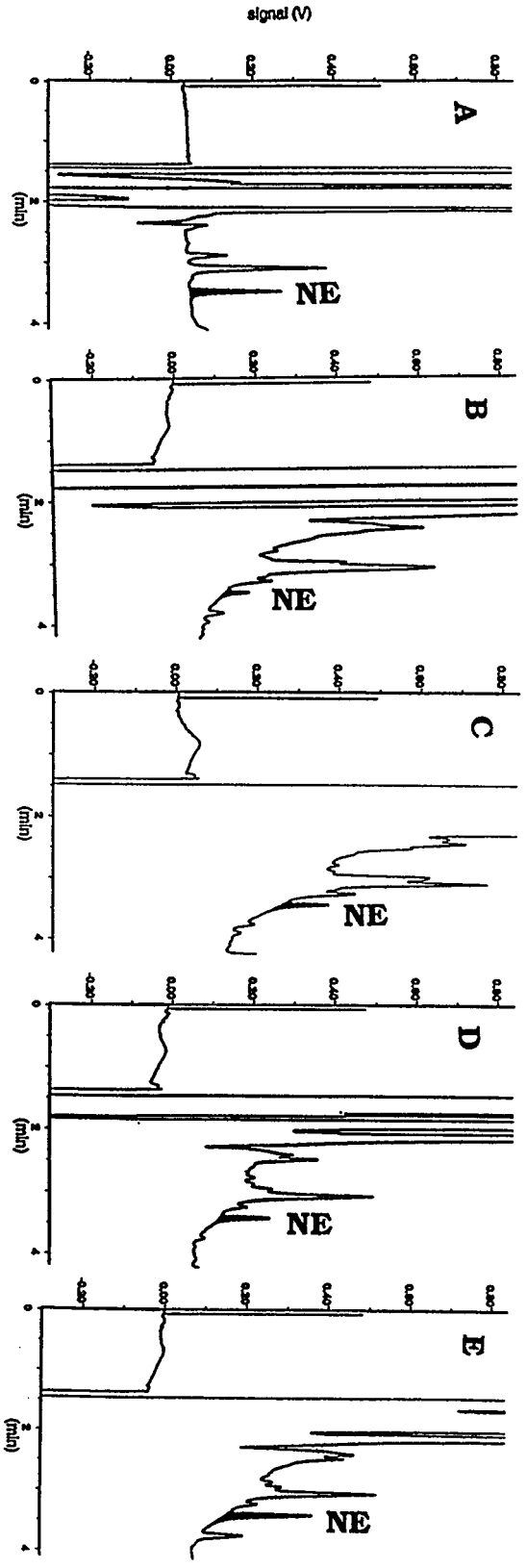


**Fig. 2.** Chromatograms of NE in standard solution and dialysate samples. The synthetic NE peak in the standard (10 pg) was symmetrical and had a retention time of 3.45 min (A). Panels B and D show representative basal levels of NE present in dialysates collected simultaneously from the AMYG and HP, respectively, of one rat. Panels C and E illustrate the increase in the level of NE in dialysates from the AMYG (C) and HP (E) after i.v. infusion of 0.09 mg/kg nicotine in the same rat.

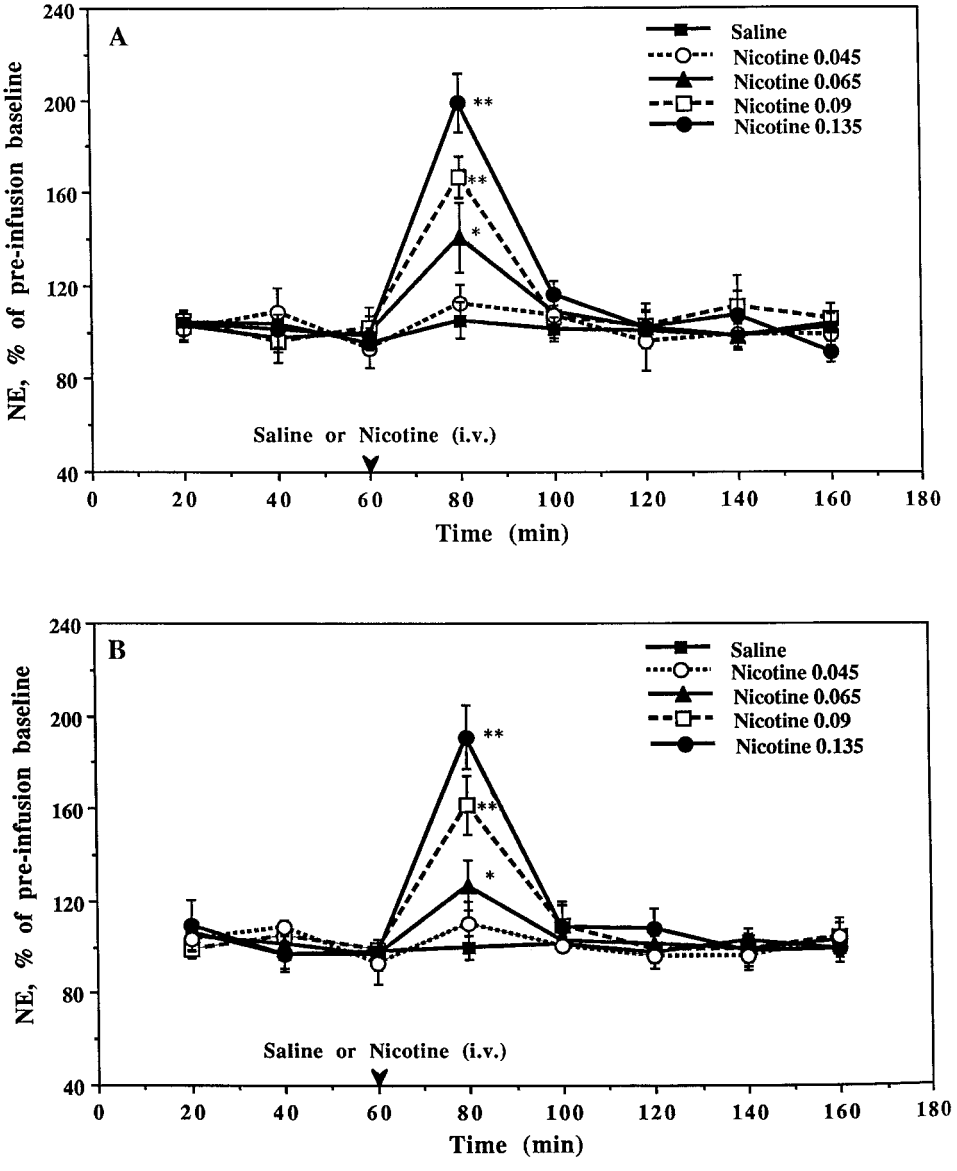


**Fig. 3.** Nicotine stimulates NE release in the AMYG (A) and HP (B) in a dose-dependent manner. NE levels are expressed as percent of pre-infusion basal levels (see "Materials and Methods"). The basal levels of NE (mean ± S.E.M.) in the saline group were 2.4 ± 0.7 pg/16 μl in the AMYG and 5.3 ± 0.9 pg/16 μl in the HP. Panel A illustrates the time course for NE release in the AMYG in response to i.v. infusions of nicotine (0.045 mg/kg per 30 sec, 0.065 mg/kg per 44 sec, 0.09 mg/kg per 60 sec, 0.135 mg/kg per 90 sec). The peak levels of NE in response to nicotine doses of 0.065 mg/kg or greater were significantly greater than saline levels. Panel B demonstrates the time course for NE secretion in HP in response to i.v. infusions of nicotine. The results are similar to those observed in the AMYG. * $P < .05$, ** $P < .01$, compared with saline ($n = 6$ rats/group).

inhibition of 62%. MLA at doses of 5.4 nmol or greater dose-dependently blocked NE release; its $IC_{50}$ was 5 nmol and NE secretion was maximally inhibited by 60%. Lower doses of MLA (0.4–1.8 nmol) had no effect on nicotine-induced NE release in the AMYG.

Figure 4B and table 1 show that nicotine-induced NE release in the HP also was blocked by Mec, DHβE and MLA, with potencies and efficacies similar to those observed for the AMYG. The $IC_{50}$ values were 6 nmol for Mec, 15 nmol for DHβE and 5 nmol for MLA. Similar to the inhibition seen in the AMYG, Mec also was more efficacious at inhibiting NE secretion in the HP (maximal inhibition, 87%) than DHβE and MLA.

In contrast, α-BTX had no effect on NE release in either of these regions (fig. 5; P = .621 for AMYG and .473 for HP, compared with CSF/nicotine). Higher doses of α-BTX could not be evaluated because they frequently produced considerable agitation, as indicated by gasping, running and jumping.

The anatomical specificity of NAchR antagonists for the brainstem, after their administration into the cerebral aqueduct, was evaluated in two ways. First, the potential diffusion of a large fraction of the delivered dose of an antagonist into the systemic circulation was assessed by injecting the $IC_{50}$ dose of Mec or DHβE (from table 1) into the jugular vein before infusing 0.09 mg/kg nicotine. The data presented in table 2 show that nicotine-induced NE secretion in both the AMYG and HP was unaffected. Second, the hypothetical delivery of NAchR antagonists *via* the CSF circulation to presynaptic NAchR in rostral brain sites was evaluated by administering Mec or DHβE directly into the AMYG and HP through a microdialysis probe. Again, no inhibition of nicotine-induced NE secretion was observed (table 2). Therefore, it appears that brainstem NAchRs were targeted by the antagonists administered into the cerebral aqueduct.

## Discussion

NE neuronal cell bodies are localized exclusively within brainstem regions (designated "A" by convention) and project rostrally throughout the brain. Approximately 90%

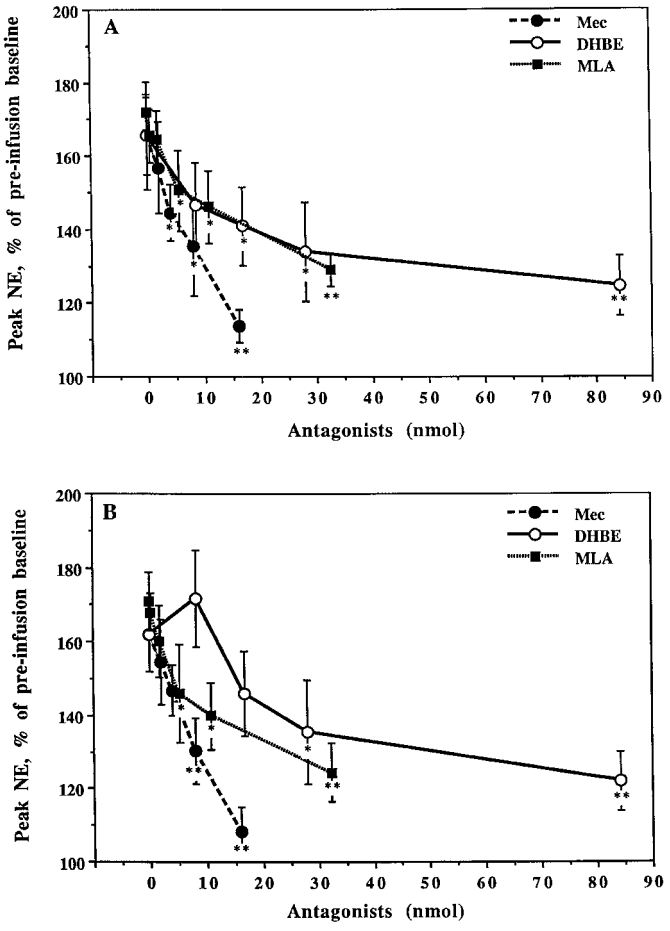


**Fig. 4.** Dose-dependent blockade of nicotine-induced NE release in the AMYG and HP after microinjection of antagonists into the cerebral aqueduct. Peak NE levels were measured in the samples collected 20 min after infusion of nicotine (0.09 mg/kg i.v.), and were expressed as percent of pre-infusion basal levels. The basal NE value (mean ± S.E.M.) in CSF/nicotine group for AMYG (A) was 1.8 ± 0.4 pg/16 μl in experiments performed with Mec and DHβE, and was 2.2 ± 0.5 pg/16 μl in those experiments performed with MLA. In the HP (B), the basal NE value was 4.3 ± 0.9 pg/16 μl for experiments with Mec and DHβE, and 4.8 ± 0.8 pg/16 μl in the MLA studies. Similar doses of Mec, DHβE and MLA inhibited nicotine-induced NE release in the AMYG and HP. * $P < .05$, ** $P < .01$, compared with microinjection of CSF ("0 nmol") into the cerebral aqueduct followed by nicotine (0.09 mg/kg i.v.; $n = 5$ rats/treatment).

TABLE 1

Nicotinic antagonists injected into cerebral aqueduct block nicotine-stimulated NE release in AMYG and HP: approximate $IC_{50}$ values and efficacies

| Antagonists | AMYG | | HP | |
|---|---|---|---|---|
| | Approximate $IC_{50}$ | Maximal Inhibition | Approximate $IC_{50}$ | Maximal Inhibition |
| | *nmol* | % | *nmol* | % |
| Mec | 6 | 80 | 6 | 87 |
| DHβE | 8 | 62 | 15 | 63 |
| MLA | 5 | 60 | 5 | 66 |

of the NE neurons are located in the LC (A6 region, Holets, 1990; Aston-Jones *et al.*, 1995), with most of the remaining neurons found in the NTS-A2 region of the dorsal medulla or in the ventromedullary A1 region (Holets, 1990). The AMYG receives input from the LC in rostral brainstem (Fallon *et al.*, 1978; Jones and Yang, 1985; Petrov *et al.*,

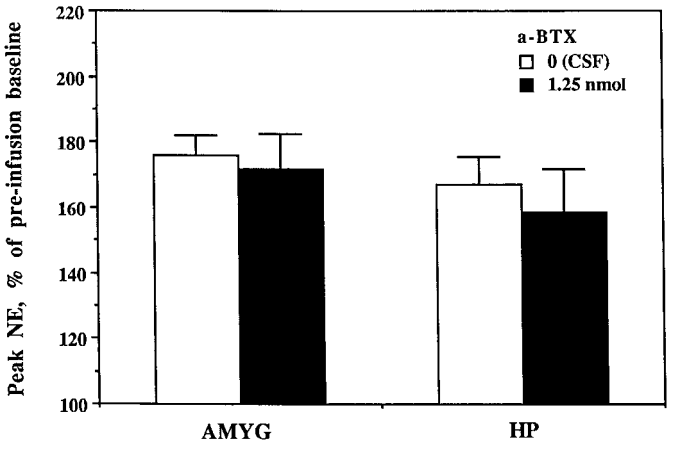


**Fig. 5.** Lack of antagonism by α-BTX injected into the cerebral aqueduct on nicotine-stimulated NE release in the AMYG and HP. No significant difference occurred between rats receiving a microinjection of CSF (CSF/nicotine) and those receiving 1.25 nmol of α-BTX 15 min before an i.v. infusion of 0.09 mg/kg nicotine ($n = 4$/group).

1993) and both the NTS and A1 in caudal brainstem (Kalia *et al.*, 1985; Zardetto-Smith and Gray, 1990; Petrov *et al.*, 1993; Roder and Ciriello, 1993), whereas the HP receives NE input primarily from LC (Aston-Jones *et al.*, 1995). Brainstem NAchRs have been identified in studies demonstrating *in situ* hybridization for subunit mRNAs (Wada *et al.*, 1989; Marks *et al.*, 1992) or high-affinity binding of radiolabeled agonists ($^3$H-nicotine and $^{125}$I-α-BTX; Clarke *et al.*, 1985; Maley and Seybold, 1993). Although the precise cellular localization of NAchRs to NE somata or other neurons has not been reported, the brainstem appears to be the primary site of action of nicotine that leads to the activation of NE neurons.

Nicotinic antagonists have been used to show that the brainstem mediates the effects of systemic nicotine on NE secretion. Antagonists administered intraparenchymally (into LC, Mitchell 1993) or *via* the aqueduct (fig. 4) and fourth ventricle (Fu *et al.*, 1997) effectively block nicotine-stimulated NE secretion in rostral targets (HP, AMYG and PVN, respectively). Diffusion of the antagonists from the CSF to the blood at sufficient concentration to act at unspecified sites in the periphery is an unlikely possibility. This possibility was eliminated by experiments in which the $IC_{50}$ concentrations of Mec or DHβE were administered i.v. (table 2); nicotine-stimulated NE was unaffected. It also is possible that antagonists delivered into the aqueduct and/or fourth ventricle could gain access to regions of the brain other than the brainstem and block the effect of systemic nicotine. Such a site could be the HP itself, which is immediately accessible from the ventricular system. The HP contains multiple NAchRs (Wada *et al.*, 1989, Flores *et al.*, 1992; Hill *et al.*, 1993), some of which appear to be presynaptic, because synaptosomal preparations released NE in response to nicotine (Clarke and Reuben, 1995; Vizi *et al.*, 1995). However, when Mec was dialyzed directly into the HP, NE secretion in response to s.c. nicotine was unaffected (Mitchell, 1993). In the present investigations, when Mec or DHβE were dialyzed into the HP at concentrations calculated to approximate brainstem $IC_{50}$ doses, NE secretion elicited by i.v. nicotine was unchanged (table 2). These findings do not preclude, however, an action of nicotine directly on Mec- or DHβE-

TABLE 2
Nicotine-stimulated NE release is not inhibited by Mec or DHβE, administered i.v., or by microdialysis probe into either AMYG or HP

| Delivered *via* | NE Levels (% of basal) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | AMYG | | | | HP | | | |
| | CSF | Mec | DHβE | P value | CSF | Mec | DHβE | P value |
| i.v. | 172.7 ± 8.8 | 163.8 ± 12.5 | 168.4 ± 10.4 | .692 | 164.2 ± 11.7 | 155.8 ± 13.8 | 170.6 ± 8.6 | .371 |
| probe | 170.2 ± 12.0 | 164.0 ± 8.8 | 162.8 ± 8.4 | .595 | 167.9 ± 10.8 | 172.9 ± 11.7 | 158.9 ± 7.4 | .460 |

insensitive NAchRs located presynaptically on NE terminals within the HP itself (or AMYG).

The present study demonstrates that systemic nicotine stimulated NE release in both the AMYG and HP with similar potency and efficacy. This similarity could be explained by the common origin of the NE input to these structures. In contrast, NE secretion in the PVN is sensitive to a dose of nicotine (*i.e.,* 0.045 mg/kg i.v.) that was ineffective in the AMYG or HP (Fu *et al.*, 1997). This difference may be because the NTS provides 80% of the NE afferents to the PVN, whereas only 10% arise from the LC or the A1 (Sawchenko and Swanson, 1982; Cunningham and Sawchenko, 1988). Indeed, the LC and A1 have been shown to be less sensitive to systemic nicotine than the NTS in studies demonstrating dose-dependent stimulation of cFos expression (Matta *et al.*, 1993, Valentine *et al.*, 1996). The presence of NAchRs of differing affinity may account for this regional sensitivity to nicotine.

Two approaches have been used to demonstrate the presence of multiple NAchRs in the brainstem. *In situ* hybridization histochemistry has been used to localize mRNA transcripts for different rat *alpha* and *beta* subunits (Wada *et al.*, 1989; Marks *et al.*, 1992), and receptor autoradiography in rats and cats has shown high-affinity binding of both $^{3}$H-nicotine and $^{125}$I-α-BTX (Clarke *et al.*, 1985; Maley and Seybold, 1993). α-BTX binding has been found in both the LC and NTS (Clarke *et al.*, 1985; Maley and Seybold, 1993), which suggests the presence of *alpha*-7 subunits. This is based on evidence showing that most α-BTX-binding NAchRs contain *alpha*-7 subunits in the mouse brain (Stitzel *et al.*, 1996). Although *alpha*-4 *beta*-2 is the dominant configuration of mammalian brain NAchRs with high affinity for $^{3}$H-nicotine (Flores *et al.*, 1992), *alpha*-4 transcripts have not been found in the LC and are only expressed at very low levels in the NTS (Wada *et al.*, 1989). The presence of specific NAchR subunits in the A1 region has not been reported. In contrast, the mRNAs for *alpha*-2, *alpha*-3, *beta*-2 and *beta*-4 have been localized in both the rat LC and NTS (Wada, *et al.*, 1989). Therefore, it appears that multiple NAchR subtypes exist in both the LC and NTS.

*In vitro* oocyte preparations expressing specific combinations of NAchR subunits have been used to define the relative efficacy and potency of the currently available nicotinic antagonists (Luetje and Patrick, 1991; Drasdo *et al.*, 1992; Cachelin and Rust, 1995; Harvey and Luetje, 1996). Based on these observations, the current study compared the relative efficacies of several nicotinic antagonists at blocking NE in response to systemic nicotine to identify pharmacologically the receptor subtypes involved. For two reasons, *alpha*-7 subunits do not appear to be involved in the stimulation of NE secretion by brainstem NAchRs. 1) Injection of α-BTX, which is highly potent and selective for *alpha*-7 subunits, did not block the effect of nicotine in either the AMYG or HP. 2) Based on *in vitro* hippocampal studies, MLA is approximately 1000- to 10,000-fold more potent at blocking *alpha*-7-mediated currents than DHβE or Mec (Alkondon and Albuquerque, 1993; Briggs and McKenna, 1996). However, as shown in table 1, the $IC_{50}$ value for blockade by MLA was not substantially less than the other two antagonists in either the AMYG or HP. This indicates that NE secretion in the AMYG and HP is not mediated by *alpha*-7-containing NAchRs in the brainstem.

Two lines of evidence obtained from published reports and the present investigations suggest that *alpha*-4 *beta*-2, the dominant high-affinity nicotine binding site in the CNS, is not likely to mediate nicotine-induced NE secretion. 1) Levels of *alpha*-4 mRNA are very low in the NTS and absent in the LC (Wada *et al.*, 1989). 2) At *alpha*-4 *beta*-2 NAchRs, DHβE is reported to be much more potent than Mec, and its efficacy is similar to MLA (Luetje *et al.*, 1990; Alkondon and Albuquerque, 1993). Table 1 shows, however, that the $IC_{50}$ value for blockade of NE secretion by DHβE was not different from Mec in the AMYG and actually was 2- to 3-fold greater than Mec in HP, providing little evidence for stimulation of NE secretion by brainstem *alpha*-4 *beta*-2 receptors. However, these findings must be interpreted cautiously in view of possible differences in the diffusion of these antagonists *in vivo*.

In contrast, involvement of *alpha*-3 *beta*-2 receptors is suggested by the similar potency that all three antagonists had for NE secretion in AMYG and HP. Oocyte transfection studies indicate that only *alpha*-3 *beta*-2 subunits demonstrate relatively similar sensitivity to Mec ($IC_{50}$ = 2.9 μM; Cachelin and Rust, 1995) and DHβE ($IC_{50}$ = 0.41 μM; Harvey and Luetje, 1996). The only report of MLA with transfected *alpha*-3 *beta*-2 receptors showed an $IC_{50}$ of 80 nM, a value approximately 2 orders of magnitude greater than its potency at *alpha*-7-containing NAchRs (Drasdo *et al.*, 1992). Moreover, MLA appears to be much more potent at *alpha*-3 *beta*-2 than at *alpha*-3 *beta*-4 (0% inhibition of apparent *alpha*-3 *beta*-4 receptors with 100 nM MLA in hippocampal cultures; Alkondon and Albuquerque, 1993). Based on these differences in the potencies of the three antagonists used in the present investigations, our observations favor the involvement of *alpha*-3 *beta*-2 in nicotine-induced NE release in the AMYG and HP.

An additional contribution from *alpha*-3 *beta*-4 subunits is suggested by the greater efficacy of Mec (80–87% blockade; table 1), which has been shown to be more effective than DHβE at *alpha*-3 *beta*-4 receptors (Alkondon and Albuquerque, 1993). Other studies with either Mec or DHβE support these observations, in that Mec was more potent at *alpha*-3 *beta*-4 ($IC_{50}$ = 0.19 μM, Cachelin and Rust, 1995) than DHβE ($IC_{50}$ = 23.1 μM, Harvey and Luetje, 1996). The involvement of both *alpha*-3 *beta*-2 and *alpha*-3 *beta*-4 NAchRs also is supported by *in situ* hybridization studies demonstrating

that *alpha*-3 is the major nicotinic agonist-binding subunit found in both the LC and NTS, whereas moderate levels of both *beta*-2 and *beta*-4 subunit mRNAs are present (Wada *et al.*, 1989). Therefore, a heterogeneous population of brainstem NAchRs, which primarily comprises *alpha*-3 *beta*-2 and *alpha*-3 *beta*-4 subtypes, may mediate nicotine-stimulated NE release in the AMYG and HP.

The ability of nicotine to stimulate the release of NE in these brain regions may underlie some of the psychoactive effects of systemic nicotine, because the noradrenergic system of the brain is involved in stress-related responses and memory function (Bremner *et al.*, 1996). Systemic nicotine induced cFos expression in amygdaloid neurons (Matta *et al.*, 1993, 1998, in press) and stimulated NE release in the AMYG in a dose-dependent manner (fig. 2). NE release in the AMYG has been reported to increase in response to immobilization stress (Beaulieu *et al.*, 1987), and AMYG activation is involved in acoustic startle and increased cardiac output (Gray, 1993). In addition, direct infusion of NE into the AMYG facilitates memory (Liang *et al.*, 1990). The AMYG is essential to working memory performance (Ohno *et al.*, 1993), in emotional memory (LeDoux, 1992; Izquierdo and Medina, 1995; Cahill *et al.*, 1996; Maren, 1996) and in regulation of the storage of memory by other brain regions (McGaugh *et al.*, 1990; Galvez *et al.*, 1996). Thus, nicotine may enhance memory functions and modulate stress responses that involve NE release in the AMYG.

The role of NE in HP memory processing is suggested by reports showing that iontophoretically applied NE induced long-term potentiation spikes in granule cells of the hippocampal dentate gyrus (Harley, 1987) and enhanced HP functions involved in memories of inhibitory avoidance and spatial habituation (Izquierdo *et al.*, 1992; Izquierdo and Medina, 1995). Studies demonstrating that systemic nicotine elicited NE release in the HP (Mitchell, 1993) in a dose-dependent manner (current study, fig. 2), as well as in the AMYG, provide a potential mechanism(s) for the memory-enhancing effects of nicotine shown in human studies (Newhouse *et al.*, 1988, 1990; Warburton, 1990).

In summary, the current study demonstrates that systemic nicotine stimulates the release of similar levels of NE in the AMYG and HP by acting on brainstem NAchRs. Pharmacological characterization indicates that brainstem *alpha*-7-containing NAchRs are not implicated; *alpha*-3-containing receptors may be involved, although additional studies are needed to evaluate that further.

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