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Allergic lung inflammation affects central noradrenergic control of cholinergic outflow to the airways in ferrets

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Wilson CG, Akhter S, Mayer CA, Kc P, Balan KV, Ernsberger P, Haxhiu MA. Allergic lung inflammation affects central noradrenergic control of cholinergic outflow to the airways in ferret. *J Appl Physiol* 103: 2095–2104, 2007. First published September 13, 2007; doi:10.1152/jappphysiol.01182.2006.—Brain stem noradrenergic cell groups mediating autonomic responses to stress project to airway-related vagal preganglionic neurons (AVPNs). In ferrets, their activation produces withdrawal of cholinergic outflow to the airways via release of norepinephrine and activation of α_{2A} -adrenergic receptors (α_{2A} -AR) expressed by AVPNs. In these studies, we examined the effects of allergen exposure of the airway (AE) with ovalbumin on noradrenergic transmission regulating the activity of AVPNs and, consequently, airway smooth muscle tone. Experiments were performed in vehicle control (Con) and AE ferrets. Microperfusion of an α_{2A} -AR agonist (guanabenz) in close proximity to AVPNs elicited more pronounced effects in Con than AE ferrets, including a decrease in unit activity and reflexly evoked responses of putative AVPN neurons with a corresponding decrease in cholinergic outflow to the airways. Although no differences were found in the extent of noradrenergic innervation of the AVPNs, RT-PCR and Western blot studies demonstrated that AE and repeated exposure to antigen significantly reduced expression of α_{2A} -ARs at message and protein levels. These findings indicate that, in an animal model of allergic asthma, sensitization and repeated challenges with a specific allergen diminish central inhibitory noradrenergic modulation of AVPNs, possibly via downregulation of α_{2A} -AR expression by these neurons.

asthma; airway-related vagal preganglionic neurons; unit discharge; α_{2A} -adrenergic receptors; guanabenz; reverse transcriptase-polymerase chain reaction; Western blotting

IN ANIMAL MODELS OF BRONCHIAL asthma and in asthmatic patients, repeated exposures to allergens and the resulting airway hyperreactivity create a stressful and potentially life-threatening condition. This response further enhances excitatory neural transmission to airway-related vagal preganglionic neurons (AVPNs) controlling cholinergic outflow to the airways. The neural pathways underlying the allergen exposure-induced centrally mediated facilitation of airway responses remain unknown.

Reflex airway narrowing is a protective response, and it is likely that recurrent exposure to noxious agents, including allergens in sensitized subjects, leads to a central nervous system primed and potentially hyperactively responsive to stimuli presented by the external environment (23). In response to allergens, the sensitized organism tends to shield the airways

to prevent lung inflammation. This defensive response to allergens can be augmented by a withdrawal of central inhibitory transmission and a resulting enhancement of excitatory input to AVPNs.

The noradrenergic system is part of the brain neural circuitry involved in responding to stress and anxiety, behavioral state control, and regulation of autonomic function (4, 14, 41). Recently, using conventional cellular labeling, transneuronal labeling techniques, ultrastructural, and molecular approaches, it has been shown that norepinephrine (NE)-containing cells project to AVPNs (15, 17). Endogenously released NE or exogenously administered moxonidine [a partial α_{2A} -adrenergic receptor (α_{2A} -AR) agonist], decreases cholinergic outflow to the airways and diminishes airway constrictive reflex responses via activation of the α_{2A} -ARs (20, 25). The α_{2A} -AR subtype functions as a heteroreceptor on nonnoradrenergic cells and their axon terminals, regulating neurotransmitter release (5, 6, 30, 42, 46), and it serves as an autoreceptor on noradrenergic neurons and axon terminals to control NE levels (1). Stress affects expression and function of both autoreceptors and heteroreceptors (11, 33). In the present study, we used central noradrenergic signal transmission as a model system to test the hypothesis that allergen exposure (AE) and repeated exposures to a specific antigen will diminish central inhibitory input to AVPNs, which, in turn, will enhance excitatory inputs to the vagal preganglionic system. The ultimate result of this inhibitory withdrawal will be a sustained increase in cholinergic outflow to the airways and airway narrowing in response to bronchoconstrictive stimuli.

To test this hypothesis, we used an in vivo ferret model and performed experiments demonstrating that AE and airway challenges with a specific antigen significantly alter AVPN unit discharge in response to lung afferent input. Additionally, AE results in an increase in tracheal tone when α_{2A} -ARs are activated with guanabenz, a potent α_{2A} -AR-agonist with a guanidine structure (9, 10, 32), in comparison to activation before allergen exposure. Although noradrenergic innervation of AVPNs did not differ between control (Con) and AE ferrets, both RT-PCR and Western blotting studies demonstrated downregulation in message and protein levels of α_{2A} -ARs in the AVPN-containing region of the ventrolateral medulla of AE ferrets compared with Con. These novel findings indicate that AE and repeated exposure to a specific antigen decreases central inhibitory noradrenergic transmission, possibly via downregulation of α_{2A} -ARs expressed by putative AVPNs.

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This loss of inhibitory input may lead to enhanced constriction in AE airways.

MATERIALS AND METHODS

Animals. The studies were performed in 59 male European ferrets, *Mustella putorius furo* (Marshall Farm, NY; 850–1,400 g). All animal experiments were conducted in accordance with the protocols approved by the Institutional Animal Care and Use Committees of both Case Western Reserve University and Howard University. Ferrets, accustomed to frequent handling, were housed on a regular day-night cycle (lights on from 0800 to 2000) at 24°C and 55% relative humidity. In the first series of experiments, we examined the effect of AE on unit discharge in response to stimulation of α_{2A} -AR by guanabenz (Sigma, St. Louis, MO) in Con ($n = 8$) and AE ($n = 13$) ferrets. In the second group, we determined effects of α_{2A} -AR activation on cholinergic outflow and airway smooth muscle tone (Con, $n = 6$; AE, $n = 7$). In the third group of animals, we examined antigen-induced modulation of AVPN innervation by NE-containing axon terminals (Con = 3; AE = 3). In addition, we measured expression of α_{2A} -AR at message (mRNA) level (Con, $n = 3$; AE, $n = 3$) and protein level (Con, $n = 3$; AE, $n = 3$) in a separate cohort of animals.

AE. Pathogen-free male ferrets were used in all experiments. Animals were randomly divided into two groups: the AE group was treated using a slightly modified version of a protocol previously described in ferrets (3), whereas the Con, receiving saline and alum, were challenged by saline inhalation. Briefly, AE was performed with intraperitoneal injections of ovalbumin (OA; Sigma grade V; 5% wt/vol, 1 ml) and $Al(OH)_3$ (20 mg) on days 1 and 15 by subcutaneous injection. Seven days after the second injection, animals were challenged by inhalation of 5% OA for 30 min on days 22 and 23. At the end, a cutaneous test was performed by intradermal microinjection of antigen (3). Twenty-four hours after the last challenge, animals were used for in vivo experiments.

Anesthesia and stereotaxic surgery. For physiological experiments, Con and AE ferrets were initially anesthetized with thiopental (25 mg/kg ip) followed by α -chloralose (70 mg/kg ip) and then tracheotomized. A tracheal tube was inserted through an incision in the caudal portion of the cervical trachea and connected to a Harvard ventilator. Subsequently, the carotid artery and a jugular vein were cannulated for measurement of arterial pressure, withdrawal of arterial blood samples, and drug administration. Ferrets were then paralyzed (galamine hydrochloride, 4 mg/kg iv) and mechanically ventilated with room air supplemented with 100% oxygen at a constant volume of 7 ml/kg delivered (frequency of 35–40 breaths/min). One ferret was ventilated with 30% oxygen-balance nitrogen. Body temperature was continuously monitored through an esophageal thermistor probe and maintained at 38.5–39.5°C by means of a heating pad. After instrumentation, ferrets were placed in a prone position in a stereotaxic frame, and the effects of α_{2A} -AR stimulation (by microperfusion of guanabenz) on putative AVPN single-unit activity were evaluated.

Extracellular AVPN unit electrophysiology. In vivo extracellular recordings were performed as previously described (32). Briefly, fine-tip (1–3 μ m) tungsten microelectrodes (5–10 M Ω at 1 kHz; Frederick Haer) were attached to a glass micropipette with a 40- μ m tip diameter and inserted unilaterally into the right or left rostral ventrolateral portion of the medulla oblongata, 3.0–3.6 mm rostral to the obex, 3.0–3.5 mm lateral to the midline, and 1.0–1.5 mm dorsal to the ventral medullary surface, by use of a dorsal approach to the brain stem. AVPNs projecting to the extrathoracic trachea and intrapulmonary airways (18, 24) are found within the region bounded by these coordinates within the ventral medulla.

As shown in Fig. 1, signals generated by these neurons were fed via high-impedance headstages to second-stage amplifiers (model P-511, Grass), filtered (30–1,000 Hz), and fed in parallel to an oscilloscope, audio monitor, and analog-to-digital computer-based acquisition sys-

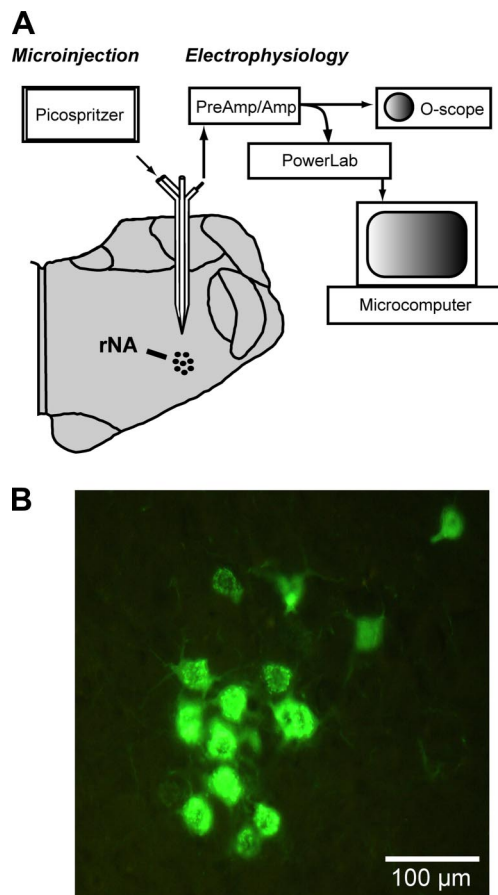


Fig. 1. Schematic of the rostral nucleus ambiguus (rNA) indicating the level and region of airway-related vagal preganglionic neurons innervating the extrathoracic trachea. A: coronal section of the ferret medulla oblongata indicating the site where a compound electrode (including a microinjection pipette and a recording electrode) is placed for single-unit recording and drug ejection (see MATERIALS AND METHODS for details). PreAmp/Amp, preamplifier/amplifier; O-scope, oscilloscope. B: an image showing retrogradely labeled airway-related vagal preganglionic neurons within the rNA 4 days after cholera toxin β -subunit (CTb) injections into the tracheal wall. Scale bar = 100 μ m.

tem (4-kHz sampling; PowerLab, AD Instruments; Colorado Springs, CO) for offline analyses. Unit activity was analyzed using the Spike Histogram add-on module in Chart (AD Instruments), which includes tunable amplitude and time windows for spike discrimination. Units recorded exhibited distinct amplitude-duration clusters. If we were unable to satisfactorily discriminate between two units, we excluded both units from analysis. Neurons in this region were spontaneously active when the ferret was artificially ventilated (volume of 7 ml/kg delivered at a frequency of 35–40 breaths/min). Unit recordings were considered to emanate from putative AVPNs if their firing rate was increased by orthodromic stimulation of rapidly adapting receptors via lung deflation ($\geq 30\%$ increase over baseline discharge). Lung deflation was induced by turning off the ventilator during the deflation phase for 10–15 s.

Response of airway smooth muscle to activation of α_{2A} -ARs expressed by putative AVPNs. In complementary studies, we measured tracheal tone as an index of parasympathetic drive to the airways. We used microdialysis for drug administration, because it allows repeated switching of subsequent concentrations of the drug delivered to putative AVPNs without repeatedly removing and replacing the cannula, minimizing the risk of damage to the region under study. After placement of a ~ 200 - μ m microdialysis probe into the right rostral nucleus ambiguus (rNA) in close proximity to AVPNs, artificial

cerebrospinal fluid (aCSF) was perfused through the probe at a maintained flow rate of 5 μ l/min with a microinjection pump (Harvard Instruments, South Natick, MA); the lag time for fluid to circulate to the tip of the probe was $\sim$ 2 min at this rate. Chemical stimulation of α_{2A} -AR at the AVPNs was performed by microdialysis of increasing concentrations of guanabenz (2, 6, 20, 60, and 200 μ g/ml) dissolved in aCSF. Before drug administration, resting tracheal smooth muscle tone in Con and AE ferrets was brought to comparable levels by adjusting the rate of the ventilator and then monitored continuously during the experiments. Increasing doses of drug were perfused after the control period. Each dose was microperfused for $\sim$ 10 min, after the initiation of microdialysis perfusion with control aCSF. At the end of each experiment, we gave atropine methylnitrate (1 mg/kg iv) to maximally relax airway smooth muscle. In these studies, no β -adrenergic airway smooth muscle-relaxing drugs were given.

Noradrenergic innervation of AVPNs. In three control and three sensitized ferrets, we analyzed the effects of sensitization and aerosol challenges on identified noradrenergic projections to the parasympathetic vagal preganglionic neurons, using a double-staining technique and confocal laser scanning microscopy (CLSM). Briefly, for labeling of AVPNs, cholera toxin β -subunit (CTb; List Biologicals, Campbell, CA) was microinjected (0.5% solution, 8 μ l total, bilaterally) into the wall of the extrathoracic trachea 3 days before initiation of aerosol exposure protocol. Twenty-four hours after the last exposure, ferrets were deeply anesthetized, perfused with 4% paraformaldehyde, and 40- μ m frozen sections of the medulla oblongata were cut. Sequential immunohistochemistry was then performed to identify noradrenergic axon terminals, using rabbit anti-dopamine- β -hydroxylase (DBH; Immunostar, Hudson, WI) as a noradrenergic marker, as previously described (17, 28). In these experiments, CTb was detected by using a fluorescein-conjugated donkey anti-goat secondary antibody, and the noradrenergic nerve endings were visualized employing a Texas red-conjugated donkey anti-rabbit secondary antibody. Neurons that exhibit CTb traits were analyzed for the number of noradrenergic axon terminal endings on somas or their proximal dendrites. We acquired confocal images (Z sectioning) at 4- μ m intervals and optical stacks of three to four images for analysis of individual identified AVPNs. Fluorescein (green) and Texas red (red) signals were acquired from the same area of the section, digitized, and stored as TIFF files. Leica provided software allows for overlay (superimposition) of images; overlap of the red and green signals generates yellow, indicating the degree to which the staining patterns arising from the different fluorophores are codistributed. In addition, in two Con and two AE ferrets, 24 h after last exposure to aerosolized ovalbumin, ferrets were deeply anesthetized, perfused, and their brains removed and processed for immunohistochemistry, in which noradrenergic fibers were visualized using DBH, and vagal preganglionic neurons in the rostral nucleus ambiguus region, were identified using choline acetyltransferase (ChAT; Chemicon International, Temecula, CA), a marker of cholinergic traits, as previously described (24). Specificity controls were obtained by replicating the experimental conditions in the absence of primary antibody.

RNA isolation and RT-PCR. Total cellular RNA was extracted from three Con and three AE ferret medullas [we punched a region of the rostral ventrolateral medulla including the nucleus ambiguus and the region immediately ventral (rNA)] by using the standard Trizol RNA extraction reagent (Invitrogen, Carlsbad, CA). The contaminating genomic DNA in the RNA samples was digested using DNaseI (Ambion, Austin, TX), and total cellular RNA was reverse transcribed using a Retroscript kit and amplified with DNA polymerase (Ambion). Briefly, 1.5 μ g of total RNA was added to a mixture containing reverse transcription buffer, dNTP, random decamers, and Moloney murine leukemia virus reverse transcriptase in a 40- μ l reaction volume. The reverse transcription was allowed to continue at 44°C for 1 h followed by termination at 92°C for 10 min. Subsequently, one-tenth of the reaction volume was amplified by using 250 nM α_{2A} -gene-specific degenerate primers. Degeneracy of the primers

was based on comparison with human, chimp, mouse, rat, and dog genomes. The multiple sequences were aligned by the ClustalW program run by the European Bioinformatics Institute. Because there is degeneracy in the primers; a touchdown PCR protocol was employed. The touchdown PCR cycling profile, using a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA), was as follows: one cycle at 94°C for 5 min, two cycles at 94°C for 30 s, 64°C for 30 s, 72°C for 45 s followed by a decrease of 2°C every second cycle in the annealing temperature until a "touchdown" at 58°C, at which temperature 30 cycles are carried out. A final extension cycle was at 72°C for 7 min. The housekeeping gene, β -actin, was used as an internal loading control, and both α_{2A} -AR and β -actin genes were amplified in the same tube. A 10- μ l aliquot of each sample was electrophoresed on a 2.0% agarose gel containing 0.5 μ g/ml ethidium bromide in Tris-acetate-EDTA buffer. As a negative control, 0.5 μ g of RNA extract was added directly to the PCR cocktail as a template for amplification. Contamination from genomic DNA would be detected as amplified DNA, and none was detected, ruling out contamination of the samples. For mRNA expression for RT-PCR, we used ferret α_{2A} -AR oligonucleotide transcript of 250-bp size [forward: 5'-AGGCCATMGAGTACAACCTG-3'; reverse: 5'-TAGATRCGCACRTAGACCAG-3'], and β -actin transcript of 638 bp [forward: 5'-AACCCCAAGGCCAACCGTG-ARAAG-3'; reverse: 5'-CTGGGWWGCCAGGGCAGTRATCT-3'].

Western blotting for α_{2A} -AR protein. Total lysates from ferret rNA brain region were extracted using mammalian tissue lysis buffer (Sigma). The lysis buffer contained a dialyzable mild detergent (bicine, 150 mM NaCl) and complete protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). Seventy-five micrograms of total protein was resolved by electrophoresis on a 12% XT bis-Tris pre-cast gel (Bio-Rad, Hercules, CA). The electrophoresed proteins were transferred to Nitrocellulose membrane (Bio-Rad) and probed with rabbit polyclonal anti- α_{2A} -AR antibody (Affinity Bioreagents, Golden, CO) (1:500 dilution from 1 mg/ml stock). The antibody was raised against a conserved peptide from human, mouse, rat, and pig (residues 218–235) within the third intracellular loop of the α_{2A} -AR. Proteins were visualized using a donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Little Chalfont, UK), and a chemiluminescence detection system (Amersham Pharmacia Biotech). The intensity of the signals in Con ($n = 3$) and AE animals ($n = 3$) were expressed as a percentage of the loading controls. The Ponceau staining record was used to correct errors introduced during the gel loading and gel transfer processes. Specifically, the predominant protein band with the largest molecular mass ($\sim$ 25 kDa), our internal loading control was used to correct the measurements. The average density of the 25-kDa Ponceau-stained and 40-kDa α_{2A} -AR band were calculated using the gel analyzer options of ImageJ software (available from the National Institutes of Health at <http://rsb.info.nih.gov/ij/>). The density readings were corrected by the differences in protein content.

Data collection and analysis. We analyzed records from physiology experiments to determine the effect of α_{2A} -AR activation by local microperfusion of guanabenz on putative AVPN unit discharge and on airway smooth muscle tone. Changes induced by guanabenz administration were calculated relative to the peak frequency of the unit discharge during baseline (before deflation or guanabenz administration). Because putative AVPNs may exhibit phasic discharge, we normalized the frequency data against the peak discharge seen over five epochs of activity (10 s per each epoch) before the first lung deflation; thus the values reported for baseline are not 100% and reflect the variability seen in each unit. In addition, we calculated changes relative to maximal relaxation of the airways following atropine methylnitrate administration. The DBH-labeled axon terminals ending on CTb- or ChAT-positive vagal preganglionic cells were counted and the average number of "contacts" per section of individual neuron were collated. We quantified the intensity of α_{2A} -AR mRNA and protein

signals for both Con and AE ferrets in absolute units (number of pixels in the region of interest). Statistical comparisons between Con and AE ferrets were made by using the Student's *t*-test or one-way ANOVA (significance at $P \leq 0.05$).

RESULTS

In tested AE ferrets, the intradermal administration of OA solution induced a round cutaneous infiltration of 7–25 mm in diameter. In Con ferrets, skin responses were negative. In one nonsensitized (Con) animal, a cutaneous bleb ~3 mm in diameter formed. In these ferrets, no histological studies were performed to determine AE-induced airway inflammatory changes.

Response of putative AVPN single unit discharge to stimulation of α_{2A} -AR. Figure 2 provides individual examples and summary data of unit responses to guanabenz administration and lung deflation in Con and AE animals. In these experiments, to determine whether sensitization and repeated airway challenges affect the response of putative AVPNs to activation of α_{2A} -ARs, we used pressure ejection to administer picomole amounts of guanabenz, the specific α_{2A} -AR agonist, dissolved in aCSF (100 nl of guanabenz acetate; 290 $\mu\text{g}/\text{ml}$). Guanabenz was administered following identification of putative single units as presumptive AVPNs, on the basis of their response to rapidly adapting receptor activation by lung deflation (33).

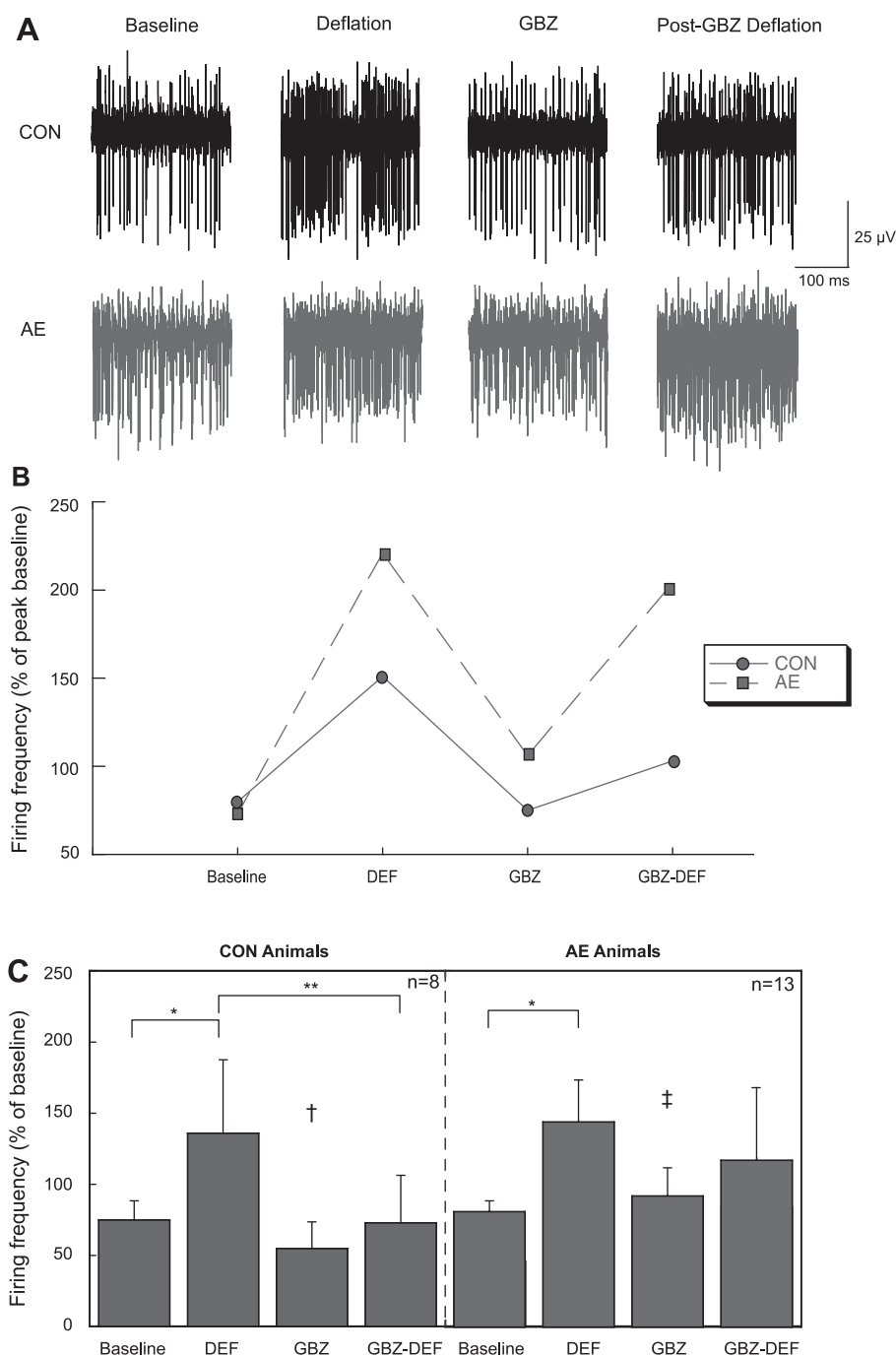


Fig. 2. Effect of α_{2A} -adrenergic receptor (α_{2A} -AR) activation on putative airway-related vagal preganglionic neuron unit activity. **A:** examples of unit discharge in vehicle control (Con; top) and allergen-exposed (AE; bottom) animals during baseline, deflation, after guanabenz injection (GBZ), and during deflation after guanabenz (GBZ-DEF). Multiple units can be seen in each trace. **B:** firing frequency is plotted for a single unit in both Con and AE animals (from A). **C:** summary data for Con ($n = 8$) and AE ($n = 13$) unit data. In both Con and AE ferrets, deflation caused a significant increase ($*P < 0.05$) in unit firing frequency (here expressed as percentage of baseline maximum). In Con animals, guanabenz significantly attenuated unit discharge compared with baseline ($\dagger P < 0.05$) and during lung deflation ($**P < 0.05$). In AE ferrets, administration of guanabenz did not attenuate the unit discharge; instead it was slightly but significantly increased ($\dagger P < 0.05$), and the lung-deflation response was not significantly attenuated by guanabenz administration.

There was no significant difference in the baseline firing frequency for Con ($n = 8$ units) vs. AE ($n = 13$ units) animals (45.2 ± 45.3 vs. 45.1 ± 29.1 Hz, respectively). Lung deflation significantly increased unit firing rate in Con ($136 \pm 51\%$; $P < 0.05$) and in AE animals ($144 \pm 29\%$; $P < 0.05$). Before drug injection, no differences were observed in arterial blood gases [arterial PO_2 (PaO_2), arterial PCO_2 (PaCO_2)] and pH between Con and AE ferrets [Con $\text{PaO}_2 = 379 \pm 38$ Torr; AE $\text{PaO}_2 = 381 \pm 48$ Torr; Con $\text{PaCO}_2 = 41 \pm 2$ Torr; AE $\text{PaCO}_2 = 42 \pm 1$ Torr; Con pH = 7.29 ± 0.02 ; AE pH = 7.31 ± 0.04 ; $P > 0.40$; guanabenz application (1 mM; 100 nl/site)] before lung deflation, unit discharge was again recorded. Guanabenz significantly attenuated firing frequency in Con animals ($55 \pm 18\%$; $P = 0.04$) but postguanabenz discharge was significantly higher in AE animals ($92 \pm 19\%$; $P < 0.05$, $n = 13$) compared with baseline. Lung deflation was repeated, and the postdeflation change in unit firing frequency was recorded. In Con ferrets, administration of guanabenz significantly diminished the increase in poststimulus discharge of AVPNs to lung deflation ($73 \pm 33\%$; $P < 0.05$). In AE animals, the same dose of guanabenz did not significantly alter the unit's response to lung deflation compared with the preguanabenz deflation ($117 \pm 51\%$; $P = 0.33$).

Response of airway smooth muscle tone to stimulation of α_{2A} -ARs associated with AVPNs. In complementary studies, we stimulated α_{2A} -ARs in the region containing AVPNs and evaluated central cholinergic outflow using tracheal tone as an index of parasympathetic drive to the airways. In animals ventilated with 100% oxygen at a frequency and tidal volume providing PaCO_2 for ~ 10 – 12 Torr above the threshold for changes in airway smooth muscle tone, PaO_2 was above 300 Torr. Differences between groups in PaCO_2 and pH were insignificant (for all compared parameters $P > 0.50$). On average, in Con group PaCO_2 was 42 ± 1.5 Torr and in AE ferrets 41 ± 2 Torr, with an arterial pH of 7.36 ± 0.02 and 7.33 ± 0.03 , respectively. In Con animals, before microperfusion of increasing concentrations of guanabenz, baseline tracheal tone measured as a pressure in a bypassed segment of extrathoracic trachea (Ptseg) was adjusted to be comparable to Ptseg recorded in AE ferrets (Ptseg in Con: 23.3 ± 2.1 cmH₂O, in AE: 24.6 ± 1.9 cmH₂O; $P = 0.66$).

In both Con ($n = 6$) and AE ($n = 7$) ferrets, increasing concentrations of guanabenz caused a progressive decrease in Ptseg. However, as shown in Fig. 3, at any given dose above 6 $\mu\text{g/ml}$ (log of the dose concentration) for each GBZ dosage is presented in Fig. 3), the decrease in Ptseg in AE ferrets was less than in Con ferrets, reaching statistical significance at 60 $\mu\text{g/ml}$ ($P < 0.05$) and 200 $\mu\text{g/ml}$ ($P < 0.05$). When, in Con and AE ferrets, dose-dependent responses to guanabenz were analyzed by two-way ANOVA, significant differences between groups were detected ($P < 0.05$). Nonlinear curve fitting to a logistic equation (36) indicated that the estimated maximal effect of guanabenz was greater in Con ferrets than in AE ferrets ($-9.8 \pm 0.8\%$ vs. $-5.0 \pm 0.5\%$). The potency of guanabenz, as indicated by the EC_{50} value, was not affected by AE (14 ± 2.8 $\mu\text{g/ml}$ in Con vs. 11 ± 2.8 $\mu\text{g/ml}$ in AE). The Hill slope for the dose response relationship did not differ between groups, nor did it differ from the expected value of negative unity (-1.3 ± 0.31 in Con vs. -1.9 ± 0.8 in AE).

When changes in tracheal tension were presented relative to the atropine-induced decrease in Ptseg (Fig. 3B), the suppres-

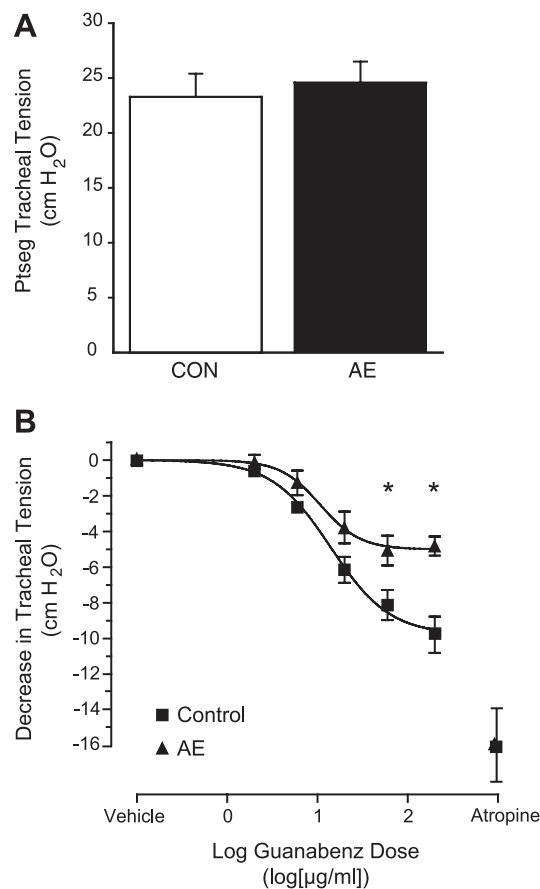


Fig. 3. Airway smooth muscle relaxation induced by stimulation of α_{2A} -ARs within the airway-related vagal preganglionic neuron region. A: baseline tracheal tone before guanabenz injection in Con and AE ferrets. B: differences in dose-dependent responses of airway smooth muscle tone between groups presented as a percentage of maximal airway relaxation (induced by systemically given atropine methylnitrate at the end of the experiment) in response to a given dose of guanabenz. Curves were constructed by taking the log of the guanabenz dose (log[$\mu\text{g/ml}$]) and graphing the resulting changes in tracheal tone for that dose. Ptseg, pressure in a bypassed segment of extrathoracic trachea. * $P < 0.05$.

sive response of guanabenz was significantly less in AE compared with the Con animals ($P < 0.05$). However, in Con and AE ferrets, the degree of tracheal relaxation, following intravenous administration of atropine methylnitrate was not different (in Con animals Ptseg decreased to 6.9 ± 0.7 cmH₂O, whereas in AE ferrets it decreased to 8.1 ± 1 cmH₂O; $P = 0.407$). AE and challenges to antigen diminished the inhibitory effects of α_{2A} -AR activation on cholinergic outflow to the airways and, consequently, the degree of airway smooth muscle relaxation.

The baseline mean arterial pressure in Con animals was significantly lower than in AE ferrets (136 ± 5.6 vs. 159 ± 6 Torr; $P = 0.026$). However, the heart rate was higher in Con compared with AE ferrets (396 ± 8 vs. 359 ± 12 beats/min; $P < 0.05$). In Con ferrets, guanabenz microperfusion into the rNA caused an insignificant decrease in mean arterial pressure (136 ± 5.6 vs. 127 ± 6 Torr; $P = 0.29$) and the heart rate (396 ± 8 vs. 381 ± 9 beats/min; $P = 0.25$). Similarly, in AE ferrets, activation of α_{2A} -ARs by the maximally effective concentration of guanabenz lowered arterial pressure only from 159 ± 6 to 147 ± 7 Torr ($P =$

0.20), and we saw no bradycardia (359 ± 12 vs. 342 ± 9 beats/min; $P = 0.30$).

Noradrenergic innervation of AVPNs. We analyzed 136 sections ($1 \mu\text{m}$ thickness; 45 neurons) from 3 Con ferrets, and 226 sections (64 neurons) from 3 AE animals. In the Con group, 28 of 45 cells were retrogradely identified as AVPNs, and 17 of 64 neurons in AE group were also identified as AVPNs. Analysis of the identified noradrenergic projections to the AVPNs using dual labeling and CLSM (Fig. 4) showed that the number of noradrenergic axon terminal endings on somata or proximal dendrites of identified AVPNs per (AE) was essentially the same as in Con group (AE: $15 \pm 1.2/\text{section}$ vs. Con: $16 \pm 1.3/\text{section}$; means $\pm$ SE; $P = 0.61$). The number of noradrenergic nerve terminal endings per section of ChAT-positive cells, within the locale of AVPNs, was higher than in retrogradely CTb-containing neurons. However, no significant differences were observed between AE vs. Con ferrets, when we compared the number of terminals per cell section of ChAT-positive neurons (AE: $42.9 \pm 2.1/\text{section}$ vs. Con: $47.1 \pm 3.2/\text{section}$; means $\pm$ SE; $P = 0.09$).

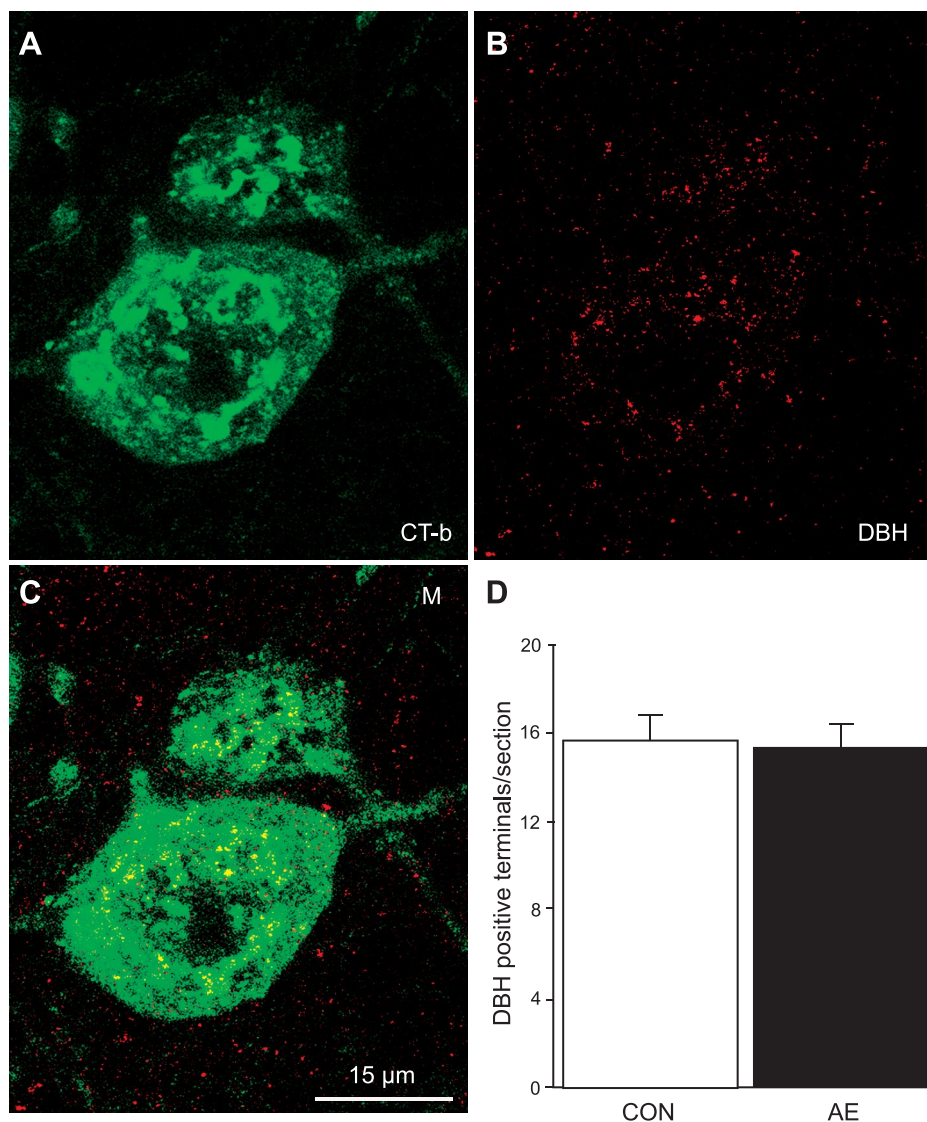
α_{2A} -AR expression in rNA region. To determine whether the decrease in AVPNs response with α_{2A} -AR activation was

correlated with loss of receptor protein, we performed RT-PCR (Con, $n = 3$; AE, $n = 3$) and immunoblot (Con, $n = 3$; AE, $n = 3$) analyses for α_{2A} -ARs from the AVPN-containing region of the brain stem. As shown in Fig. 5 (A1 and A2), RT-PCR gels for α_{2A} -AR message in representative Con and AE ferrets show receptor mRNA signals readily measurable in both Con and AE animals but reduced in AE ferrets (Con, 0.48 ± 0.13 ; AE, 0.25 ± 0.04). α_{2A} -AR protein levels (B1 and B2) were also significantly reduced in brains of AE ferrets ($P < 0.03$), as shown by pixel intensities times areas (average pixel area) as well. In the Con group, the average of α_{2A} -AR signal intensity in rNA region of the medulla oblongata was 13.6 ± 0.6 U (U = pixels $\times$ gray-scale intensity), whereas in AE ferrets it was 4.2 ± 0.3 U with a ratio of 3 ($P = 0.001$). Similarly when data were normalized for values of the internal loading control, differences between groups were statistically significant ($P = 0.001$).

DISCUSSION

We have shown, for the first time, that AE and repeated challenges to the airways suppress central noradrenergic inhib-

Fig. 4. Noradrenergic innervation of airway-related vagal preganglionic neuron regions. A: CTb-containing airway-related vagal preganglionic neuron region 4 days after CTb injection into the extrathoracic trachea of a ferret, identified by green secondary antibody (FITC). B: confocal microscopic image of noradrenergic nerve terminals within the region of the rNA expressing dopamine β -hydroxylase (DBH)-like immunoreactivity, visualized by a Texas red-conjugated secondary antibody. C: superimposed confocal microscopic images of noradrenergic axon terminals ending on the cell body and proximal dendrite of airway-related vagal preganglionic neuron region innervating the extrathoracic trachea (M = merged). D: average number of axon terminals per $1\text{-}\mu\text{m}$ -thick confocal microscopic section (total 80 sections of 27 identified airway-related vagal preganglionic neuron regions) of Con ferret and the number of axon terminals ending on airway-related vagal preganglionic neurons (total 50 sections of 16 identified airway-related vagal preganglionic neurons) of the AE animal ($P = 0.610$).



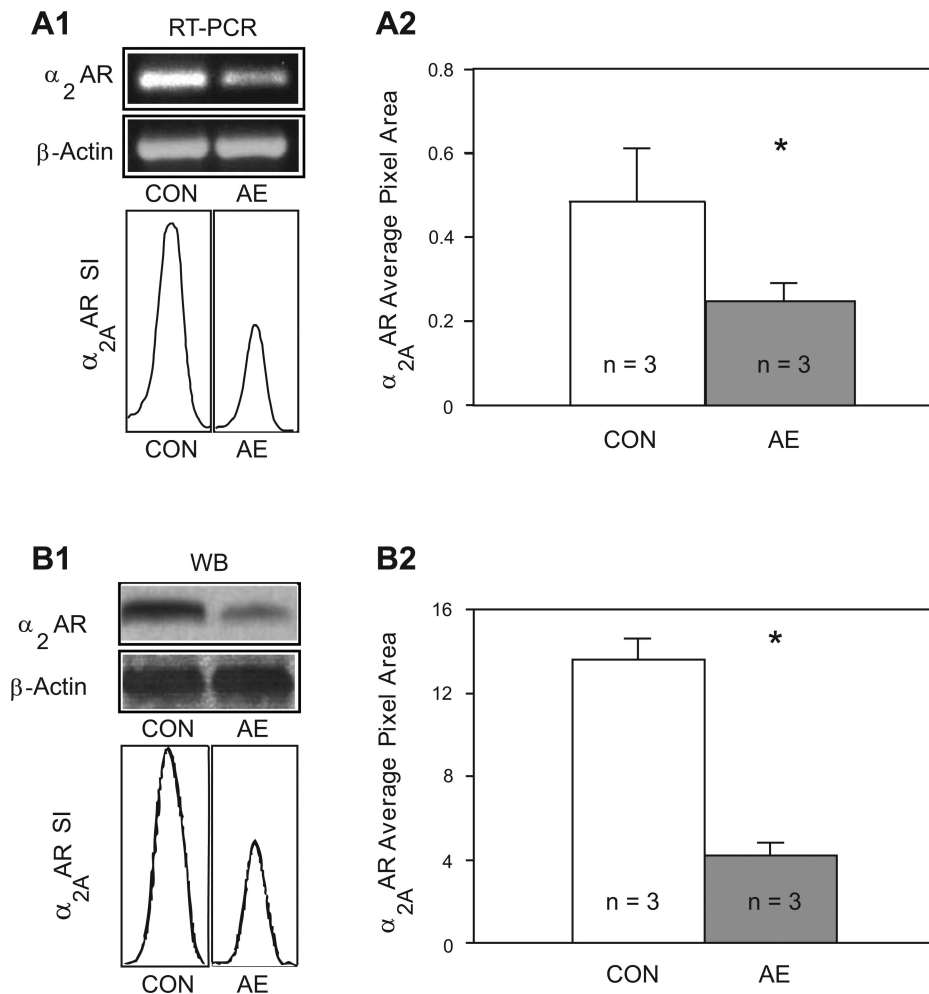


Fig. 5. A: RT-PCR and Western blots of α_{2A} -AR mRNA and protein. A1–A2: RT-PCR example and summary data. A1: total RNA was purified from fresh brain tissues dissected from rNA region of a Con ($n = 3$) and AE ($n = 3$) ferret. RT was performed using 1.5 μ g of total RNA, 5 μ mol of random decamer, and Moloney murine leukemia virus reverse transcriptase in a 40- μ l reaction mixture. Four microliters of the RT product was amplified by polymerase in the presence of 250 nmol of α_{2A} -AR-specific primer. SI, signal intensity. A2: summary data for RT-PCR presented in terms of average pixel area. * $P < 0.05$. AE animals showed a significant reduction in RT-PCR product (* $P < 0.03$). Signal of an amplified α_{2A} -AR mRNA product (250 bp) is lower (~50%) in AE than in Con ferret compared with the signal of RT-PCR product (638 bp) for β -actin mRNA. B1–B2: expression of α_{2A} -AR protein in the rNA region. B1: examples of blots in Con and AE ferret seen at 40 kDa and readings of internal loading controls recorded at ~25 kDa. W, Western blot. B2: average changes (means $\pm$ SD) between groups, presented in terms of average pixel area. * $P < 0.05$.

itory influences on AVPNs and, consequently, increase cholinergic outflow to the airways. We have also demonstrated a decrease in message and protein expression for α_{2A} -ARs in AE animals. This work has importance in our understanding of human airway disorders such as asthma.

Response of putative AVPNs to activation of α_{2A} -AR expressed by AVPNs. We have shown that α_{2A} -AR activation (on putative AVPNs) is diminished in AE ferrets. In saline aerosol-challenged animals (Con), we found that endogenously released NE (22) inhibits cholinergic outflow to the airways, resembling the effect of guanabenz on AVPNs. Taken together, these results suggest that α_{2A} -AR determines the effects of NE or its analogs on excitatory transmission to AVPNs. Previous studies have shown that increase in concentration of released NE within the rNA in Con ferrets has no effect on release, or on NE uptake, mechanisms within the rNA (22) but that, in AE ferrets, it may affect expression or sensitivity of α_{2A} -AR expressed by AVPNs.

The effects of endogenously released NE or exogenously applied NE analogs are conveyed through specific adrenergic receptor subtypes, including α_1 - and α_2 -receptor subfamilies. Each subfamily contains distinct receptor subtypes (α_{1A} , α_{1B} , α_{1D} , α_{2A} , α_{2B} , α_{2C}), coded by a separate gene, and these subtypes display characteristic tissue distribution, regulatory properties, and drug specificities (8, 25). α_{2A} -ARs are cell-

surface $G_{i/o}$ protein-coupled receptors that, when activated, lead to inhibition of adenylate cyclase activity, inhibition of presynaptic N-type Ca^{2+} channels, activation of inwardly rectifying K^+ channels, and cell hyperpolarization (7, 46). α_{2A} -ARs serve the role of heteroreceptors in contrast to α_1 -ARs (35, 39, 45), and α_2 -AR activation inhibits neuronal activity and excitatory transmission in the brain (5), partly blocking glutamate release at axon terminals (13, 38, 43).

Data from rat experiments, using selective agonists and antagonists for subtypes of α -ARs, showed that NE elicits inhibitory and excitatory effects on neurons within the dorsal motor nucleus of the vagus by decreasing or increasing the K^+ conductance through α_1 - or α_2 -ARs (12, 30). A similar biphasic effect has been seen in neurons within the nucleus ambiguus of the guinea pig (35). Similarly, activation of α_{2A} -ARs inhibits activity of neurons within the sensorimotor area that is instrumental to song production in birds (42).

Our results support and extend previous findings in ferrets (22), showing that the effects of locus coeruleus stimulation could be mimicked by guanabenz, an α_{2A} -AR agonist. Moxonidine, a partial α_{2A} -AR agonist (9), acts centrally to inhibit reflex increases in airway smooth muscle tone (19). In our hands, guanabenz reduced reflexly evoked activity of putative AVPNs more in Con than in AE ferrets. This suggests that excitability of AVPNs is reduced by activation of postsyn-

aptic α_{2A} -ARs to a greater degree in Con ferrets, indicating that allergic airway injuries downregulate noradrenergic inhibitory influences, mainly due to lowered expression of α_{2A} -ARs. These findings do not exclude the possibility that withdrawal of inhibition in AE ferrets may be (partially) due to glutamate release from axon terminals innervating AVPNs; this possibility cannot be ruled out without additional experiments because bronchomotor neurons within the nucleus ambiguus receive excitatory glutamatergic inputs (20, 21) and play a major role in providing cholinergic outflow to the airways (16). α_{2A} -ARs control glutamatergic transmission in the nervous system (5, 13, 38), suggesting a dynamic interplay between α_{2A} -AR, glutamate release, and resulting changes in cholinergic outflow. In contrast to AVPNs, noradrenergic modulation of hypoglossal inspiratory activity does not involve α_{2A} -AR-mediated inhibition and NE acts directly on α_1 -AR to increase the excitability of hypoglossal motoneurons (39, 45).

Response of airway smooth muscle to stimulation of α_{2A} -ARs expressed by AVPNs. In Con ferrets, activation of the α_{2A} -AR expressed by AVPNs induced a withdrawal of cholinergic outflow to the airways in a dose-dependent fashion. This response was slightly reduced in AE animals, paralleling the decrease in putative AVPN responses to guanabenz and the decreased expression of α_{2A} -AR by AVPNs. The decrease in expression of the α_{2A} -AR protein likely leads to a loss of inhibitory modulation that then facilitates central cholinergic output to the airways elicited by bronchoconstrictive stimuli.

The differences between putative AVPN response and smooth muscle tone in Con and AE ferrets in response to guanabenz could be due to changes in arterial pressure induced by activation of α_{2A} -ARs (29). Microperfusion of guanabenz into the rNA region in both groups caused a slight decrease in arterial pressure. This could augment rather than decrease airway smooth muscle tone and, correspondingly, raise rather than lower tracheal pressure (40). Furthermore, in these studies, the tracheal tone was adjusted to comparable levels between Con and AE ferrets, and airway constriction was reversible in both groups (as shown by the decrease in Ptseg) following intravenous administration of atropine methylnitrate, thus reducing extraneous factors that can modulate airway responses and confound our results.

Effects of AE and repeated exposures to antigen on noradrenergic innervation of AVPNs. The quantitative analysis of noradrenergic axon terminals innervating cholinergic neurons within the rNA revealed that sensitization and repeated exposure to aerosolized antigen has no significant effect on the quantity of noradrenergic nerve endings on cholinergic neurons within the rNA, a subpopulation of which innervate the airways. Based on confocal image analysis, we cannot conclude that these noradrenergic nerve endings make direct synapses with AVPNs in the rNA. It is highly likely that the majority of these terminals end at the vicinity of these cells without making "classical contacts." This assumption is based on our laboratory's previous work showing that only a small percentage of robust catecholaminergic nerve terminals formed direct synapses with identified AVPNs (22).

We also observed differences in the number of terminals found (per section of cell) on CTb-labeled neurons compared with unlabeled cholinergic expressing cells within the rNA, although we do not know the reason for these differences. We do not believe that inefficient antibody penetration contributed

to an underestimation of terminals because our tissues were pretreated to facilitate antibody penetration and were processed at the same time. Furthermore, robust DBH labeling was observed throughout the sections. It may be that AVPNs are innervated by catecholaminergic neurons to a lesser degree than other cholinergic cells intermingled with cholinergic cells projecting to the airways. Whatever the cause of the difference, sensitization and exposure to antigen did not lead to any observable effect that can be quantified by the methods we employed in this study.

Our results do not reveal the origin of terminals or varicosities labeled by DBH immunohistochemistry ending on AVPNs. Previous studies (7) have shown that the majority of noradrenergic neurons innervating AVPNs were observed in distinct groups: along the ventrolateral margin of the pontine tegmentum (A5 cell group), dorsolateral to the midline, and beneath the fourth ventricle (locus coeruleus and subcoeruleus, A6 cell group). There is a small contribution of catecholaminergic neurons located in the ventrolateral medullary reticular formation (A1 and C1 cell groups) and in the dorsal aspect of the medulla oblongata (A2 and C2 cell groups) to noradrenergic or adrenergic innervation of AVPNs (7). Based on this and a previous study (23), it is probable that the released NE reaches extra-synaptic membrane receptors on AVPNs by diffusion, volume transmission (2), and, to a lesser extent, via direct synaptic communication.

Effects of AE and repeated exposures to antigen on expression of α_{2A} -AR by AVPNs. This study provides evidence that AE can affect the α_{2A} -ARs on the transcriptional level and at the level of the receptor protein. We have shown for the first time that α_{2A} -ARs expressed in the region where AVPNs are found are downregulated by repeated exposures to antigen, which persists for 24–48 h after the last exposure, suggesting the involvement of regulatory mechanisms in the plasticity of this transmitter system.

Physiological implications. There is increasing evidence indicating that the brain, previously neglected for its importance in bronchial asthma and chronic obstructive bronchitis, affects airway control and bronchopulmonary responses. The noradrenergic pathways are part of a convergent neural circuit through which behavioral and emotional factors interact with physiological events, influencing the severity of asthma symptoms (4, 17, 22, 44).

The firing rate of noradrenergic neurons progressively changes across the sleep-wake-arousal cycle: it diminishes in quiet sleep, is abolished when entering active rapid eye movement sleep, and is dramatically elevated during arousal (4). In humans, airway caliber also undergoes cyclic oscillations, decreasing at night and increasing during the day. These fluctuations are greatly amplified in patients with nocturnal worsening of asthma (27, 44) and may be due to withdrawal of the inhibitory influences that then trigger the cascade of events that enhances airway narrowing and promotes nocturnal worsening of airway conductivity.

In addition, fear and emotional distress may facilitate the incidence of bronchoconstrictive attacks (26). Previous receptor binding studies have shown that chronic psychosocial stress affects binding sites for α -adrenergic ligands in several brain sites (11, 33). In addition, chronic stress reduces the expression of α_{2A} -ARs on glutamatergic neurons of the brain stem (33). Because glutamate is the main excitatory neurotransmitter

involved in reflex bronchoconstriction (20, 21), the stress-induced downregulation in α_{2A} -AR expression on these neurons might augment airway bronchoconstrictive reflex responses, via increased glutamatergic drive to the vagal preganglionic neurons innervating the tracheobronchial system. Hence, AE and repeated exposures to antigen, associated with a corresponding decline in lung function should be considered stressors, affecting expression of α_{2A} -ARs. Patients suffering from bronchial asthma have a greater risk of depression (26), and α_{2A} -knockout mice are known to manifest depressive-like behavior, which is insensitive to the tricyclic antidepressant imipramine (41).

Limitations. Naturally, there are limitations to the techniques we have used in this study, and the major caveat is related to the method of single-unit recording, drug delivery, and selectivity of drugs used. In this study, we employed orthodromic stimulation (via lung deflation) to identify putative AVPNs. We investigated single-unit motor responses to stimulation of bronchopulmonary sensory receptors, which preferentially activate AVPNs, via second-order neurons within the nucleus tractus solitarius (20, 21). For example, stimulation of airway rapidly adapting receptors excites AVPNs, increasing discharge frequency and cholinergic outflow to the airways (34). However, the only indisputable way to identify AVPNs is to perform antidromic stimulation of peripheral branches of the vagus nerve that innervate the airways and to verify the projection by demonstrating collision of orthograde and anterograde action potentials. Although simple in concept, our laboratory found this difficult in practice in previous studies in ferrets (34). This may be because AVPNs have slowly conducting, nonmyelinated axons (31) in ferrets. Furthermore, many airway-related vagal preganglionic neurons have a very low rate of spontaneous activity at low levels of chemoafferent drive. These neurons have a low axonal conduction velocity and require high-intensity current for antidromic activation. Because the vagus nerve contains both afferent and efferent fibers, electrical stimulation of the vagus activates both sensory afferent fibers and motor efferent axons, and it is not easy to dissociate an antidromic component. Orthodromic activation of AVPNs does suggest that these units are involved in airway control, because in ferrets, activation of these neurons by blockade of GABA_A receptors leads to an increase in their discharge frequency and increased cholinergic outflow to the airways (34).

For drug delivery, we used pressure ejection, or microperfusion via a microdialysis probe. We found these methods to be very effective in administering drugs to discrete brain stem regions. In ferrets, AVPNs are clustered within the rNA area (18). Variations in the size of the head, leading to ejection of the drug outside of targeted regions are very small in male ferrets of comparable age and strain. All probes were within the rNA within a sphere of $\sim 700\ \mu\text{m}$ in diameter. In addition, guanabenz has been characterized as a very specific α_{2A} -AR agonist (3). Guanabenz is known to lower blood pressure by acting at a site within the gigantocellular region of the ventrolateral medulla (2). The fact that blood pressure did not fall significantly following guanabenz microperfusion indicates that this drug did not diffuse to this nearby area, implying a restricted region of the medulla was affected by the agent. The central effect of guanabenz on α_{2A} -AR receptors is inhibitory

and qualitatively different from that observed with activation of α_1 - or β_1 - and β_2 -ARs (7).

Our confocal microscopy results do not, admittedly, reveal synaptic contacts between axonal endings and AVPNs. To determine the effects of AE and aerosol challenges on noradrenergic innervation of AVPNs, we used a design that allows morphometric quantification of the single cell while avoiding double counting of axonal terminals. However, one must be cautious when interpreting these results as not all AVPNs could be retrogradely labeled. The expression of α_{2A} -AR in the rNA region of the ferret was shown, in this study, by RT-PCR, Western blot, and functional analysis. The decrease in AVPNs response to activation of α_{2A} -AR was correlated with a quantitative loss of both message and receptor protein. In our experiments, the specificity of the antibodies used was previously characterized in ferrets and successfully employed in immunocytochemistry (22). However, the obtained data do not exclusively refer to receptor protein expressed by AVPNs. Rather, we measured all α_{2A} -AR protein originating from synaptic (pre/postsynaptic), dendritic and perikaryal, as well as glial receptors within the region of AVPNs in the ventrolateral medulla. We did not use in vitro receptor autoradiography, because currently available α_2 -AR radioligands are not entirely subtype specific and will add little to the obtained results.

Summary. In conclusion, the results of this study suggest that allergic airway disorders may affect central noradrenergic inhibitory pathways, resulting in a shift from inhibitory to excitatory influences, a hyperexcitable state of the AVPNs, and centrally mediated airway hyperreactivity. Hence, drugs that potentiate central noradrenergic mechanisms via the activation of α_{2A} -ARs expressed by airway-related vagal preganglionic premotoneurons may represent good candidates for new treatments of airway overreactivity.

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