GABA in Nucleus Tractus Solitarius Participates in Electroacupuncture Modulation of Cardiopulmonary Bradycardia Reflex


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Abstract

Phenylbiguanide (PBG) stimulates cardiopulmonary receptors and cardiovascular reflex responses, including decreases in blood pressure and heart rate mediated by the brainstem parasympathetic cardiac neurons in the nucleus ambiguus and nucleus tractus solitarius (NTS). Electroacupuncture (EA) at P5-6 stimulates sensory fibers in the median nerve and modulates these reflex responses. Stimulation of median nerves reverses bradycardia through action of γ-aminobutyric acid (GABA) in the nucleus ambiguus, important in the regulation of heart rate. We do not know if the NTS or the neurotransmitter mechanisms in this nucleus participate in these modulatory actions by acupuncture. We hypothesized that somatic nerve stimulation during EA (P5-6) modulates cardiopulmonary inhibitory responses through a GABAergic mechanism in the NTS. Anesthetized and ventilated cats were examined during either PBG or direct vagal afferent stimulation while 30 min of EA was applied at P5-6. Reflex heart rate and blood pressure responses and NTS evoked discharge were recorded. EA reduced the PBG-induced depressor and bradycardia reflexes by 67 and 60%, respectively. Blockade of GABA_A receptors in the NTS reversed EA modulation of bradycardia but not the depressor response. During EA, gabazine reversed the vagally evoked discharge activity of cardiovascular NTS neurons. EA modulated the vagal evoked cardiovascular NTS cellular activity for 60 min. Immunohistochemistry using triple labeling showed GABA immunoreactive fibers juxtaposed to glutamatergic nucleus ambiguus-projecting NTS neurons in rats. These glutamatergic neurons expressed GABA_A receptors. These findings suggest that EA inhibits PBG evoked bradycardia and vagally evoked NTS activity through a GABAergic mechanism, likely involving glutamatergic nucleus ambiguus-projecting NTS neurons.
Introduction

Clinical and experimental studies suggest that acupuncture reduces hypertension, hypotension and myocardial ischemia (4, 11, 37, 41, 42, 51). Modulation of cardiovascular function and elevated sympathetic activity is effective with low frequency and voltage stimulation of electroacupuncture (EA) at specific acupoints P5-6 positioned to activate the underlying median nerves (44-46). Bilateral stimulation of P5-6 acupoints activates thinly myelinated and unmyelinated sensory fibers in the median nerve and neural pathways that project to cardiovascular-related regions in the central nervous system (CNS), which regulate autonomic activity and hence hemodynamic responses (21, 23, 39). Nuclei in the hypothalamus, midbrain and medulla process this somatic input during EA to modulate sympathoexcitatory reflex responses (26, 28, 43). Several neurotransmitter systems, including opioids and γ-aminobutyric acid (GABA) participate in the inhibitory influence of acupuncture on excitatory cardiovascular responses (13, 17, 43, 46). The activity of presympathetic neurons in the rostral ventrolateral medulla (rVLM) and nucleus raphé pallidus (NRP) are modulated by EA applied to P5-6 acupoints (27, 28). Thus, stimulation at P5-6 exerts strong cardiovascular actions by reducing reflex elevations in blood pressure (41, 44). Despite this large body of evidence indicating that EA modulates elevated blood pressure through its inhibitory action on sympathetic activity (4, 9, 21-28, 39, 41, 43-46, 46, 52-54), there is less information available about its actions on the parasympathetic nervous system (38, 42, 48, 49).

EA modulates cardiovascular depressor reflexes that originate from the gastrointestinal tract in the setting of hypercapnia-acidosis (38, 40). Under these conditions both spinal and vagal afferent pathways are stimulated to lower blood pressure through sympathetic withdrawal and increased parasympathetic outflow (40). Several medullary nuclei, including the caudal ventrolateral medulla (cVLM), rVLM and nucleus ambiguus contribute to the modulatory actions of EA through GABA mechanism (38).
Activation of cardiopulmonary vagal afferents by prostaglandin-E2, veratrum alkaloids, serotonin (5-HT), capsaicin or intravenous phenylbiguanide (PBG) elicits decreases in heart rate and blood pressure through the Bezold-Jarisch reflex (6, 8, 20, 20). Intravenous PBG stimulates cardiopulmonary serotonin (5-HT₃) afferent endings projecting to supraspinal regions to evoke hypotension and bradycardia. The nucleus tractus solitarius (NTS) and nucleus ambiguus innervated by these vagal afferents process the reflex response. Previous studies show that EA acts at the level of the nucleus ambiguus to modulate vagal preganglionic outflow to the heart (42). However, the influence of somatic nerve stimulation during EA on the NTS which, in turn, interacts with the nucleus ambiguus during stimulation of cardiopulmonary reflex responses in the NTS is uncertain. The purpose of the present study therefore was to evaluate the role of the NTS in EA modulation of the cardiopulmonary cardioinhibitory reflex.

Neurons in the intermediate NTS are involved in cardiovascular regulation. GABAergic neurons interspersed throughout the NTS co-ordinate autonomic motor outflow through the nucleus ambiguus to the heart (19, 42). Activation of GABA receptors in the NTS increases heart rate (32). Sved et al. reported that GABAergic interneurons in the NTS modulate baroreflex function (3, 35, 35). Degtyarenko and Kaufman showed that barosensitive neurons in the NTS receive input from somatic afferents (10). Thus, the current study investigated the role of GABA receptors in the intermediate NTS in neurons receiving convergent input from vagal and somatic afferents as well as baroreceptor input. We hypothesized that somatic stimulation during EA (P5-6) modulates the cardiopulmonary inhibitory responses through GABAergic mechanism in the NTS.

Materials and Methods

Anatomical studies

Retrograde tracing

All procedures were carried out in accordance with the Society for Neuroscience and the National Institutes of Health guidelines and animal use and care committee at the
University of California, Irvine. The minimum possible number of rats (n=5) was used to obtain reproducible results in this study. In addition, every effort was made to minimize discomfort and suffering. Surgical and experimental protocols were approved by the Animal Use and Care Committee at the University of California, Irvine. Adult male Sprague-Dawley rats (350-500 g) were used for microinjection of a retrogradely transported microsphere tracer into the nucleus ambiguus to evaluate direct projections from the NTS to the nucleus ambiguus, as described in detail in our previous studies (23). Ketamine/xylazine (80/12 mg/ml, Sigma) were used to induce (0.3-0.4 ml, im) and maintain (0.1-0.2 ml, im) anesthesia in the animals. Body temperature was monitored with a rectal probe and was maintained at 37 °C. Heart rate and oxygen saturation were monitored using a pulse oximeter (Nonin Medical, Inc. Plymouth, MN USA). Following induction, rats were positioned in a stereotaxic apparatus (David Kopf Instruments). Under aseptic conditions, a one inch incision was made to expose the skull. A burr hole (4 mm diameter) was made in the bone so that a glass micropipette could be inserted using the following coordinates: 13.20–14.28 mm caudal from the bregma, 1.8–2.2 mm from the midline, and 6.2-6.5 mm deep from the dural surface (30). One hundred nanoliters of a retrogradely transported tracer, rhodamine-labeled fluorescent microspheres in suspension (0.04 μm, Molecular Probes, Eugene, OR), were injected into the nucleus ambiguus through a glass micropipette. The wound was sutured shut. Buprenorphine (0.5 mg/kg, im) and penicillin (7,500 units/kg, im) were administered prior to recovery. Microspheres were transported during a 7- to 10-day recovery and maintenance period.

Terminal procedures occurred 7 to 10 days after administration of the retrograde tracer. Rats were re-anesthetized with ketamine/xylazine, as described above. After tracheotomy and intubation, the cannulation and monitoring of vital signs were similar to the procedures described below for cats. Animals were stabilized for 2 h. They then were anesthetized deeply with a large dose of the ketamine/xylazine (0.5–0.7 ml, im). Transcardial perfusion was performed using 500 ml of 0.9% saline solution followed by 500 ml of 4% paraformaldehyde. The medulla oblongata was harvested and sliced in coronal sections (30 μm) using a cryostat microtome (Leica CM1850 Heidelberger...
Brain sections were placed serially in cold cryoprotectant solution and were used for immunohistochemical labeling as described below and for identifying sites of microsphere tracer injection. In this study, free-floating sections were used for labeling.

Immunohistochemical staining

We conducted double-fluorescent immunohistochemical labeling for vesicular glutamate transporter 3 (VGLUT3, a potential marker for glutamatergic neurons) + glutamic acid decarboxylase isoform 67 (GAD67, a marker for GABAergic neurons) or GABA<sub>A</sub> receptors. After washing for 30 min (10 min x 3 times) in phosphate-buffered saline containing 0.3% Triton X-100 (PBST; pH = 7.4), brain sections were placed for 1 h in 1% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA). The sections then were incubated with two primary antibodies, including a guinea-pig anti-VGLUT3 antibody (1:500 dilution) and a mouse anti-GAD67 (1:500) or a goat anti-GABA<sub>A</sub> receptors (1:200) for 48 h at 4 °C. We used three different antibodies for the immunohistochemical reactions. The characterizations of all three primary antibodies were provided by the manufacturers. Guinea-pig anti-VGLUT3 antibody: Chemicon International, Temecula, CA, Catalog #, AB5421, Lot # 0603024959, single band on Western blots (~65kDa), pre-absorption of this antibody with the immunogen peptide (Catalog #, AG320) eliminates all immunostaining. Mouse anti-GAD67 antibody: Millipore, Temecula, CA, Catalog #, MAB5406; Lot # 2042787, No detectable cross reactivity with GAD65 by Western blot on rat brain lysate. Goat anti-GABA<sub>A</sub> receptor antibody: Santa Cruz Biotechnology, CA, Catalog #, sc-7348; Lot # G2611; single band on Western blots (43 ~ 55kDa), the immunostaining is eliminated after pre-absorption of this antibody with the immunogen peptide (Catalog #, sc-7348 P). Sections then were incubated with a coumarin-conjugated donkey anti-guinea-pig antibody and a fluorescein-conjugated donkey anti-mouse or anti-goat antibody (all 1:100; Jackson Immunoresearch Laboratories) at 4 °C for 24 h. The sections were mounted on slides and coverslipped with mounting medium (Vector Laboratories). In the immunohistochemical control studies, no stain was detected when the primary or secondary antibody was omitted.
Brain sections were scanned and examined with a standard fluorescent microscope (Nikon, E400, Melville, NY). Three epifluorescence filters (B-2A, G-2A, or UV-2A) in a fluorescent microscope were used to identify single stains appearing as green (fluorescein), red (rhodamine), or blue (coumarin) in brain sections. Sections containing the NTS were identified according to their best matched standard stereotaxic plane, as shown in Paxinos and Watson's atlas for the rat (30). After examination with the fluorescent microscope, selected sections were further evaluated with a laser scanning confocal microscope (Zeiss LSM 510, Meta System, Thornwood, NY) to confirm co-localization of two or three labels. This apparatus was equipped with Argon and HeNe lasers and allowed operation of multiple channels. Lasers of 488- and 543-nm wavelengths were used to excite fluorescein (green) and rhodamine (red), respectively. A 790-nm laser was applied for two-photon excitation of coumarin (blue). Digital fluorescent images were captured and analyzed with software (Zeiss LSM) provided with the confocal microscope. Each confocal section analyzed was limited to 0.5 µm thickness in the Z-plane. Images containing two or three colors in the same plane were merged to reveal the relationship between two and/or three labels (see Figures 1 and 2). Single-, double- and triple-labeled neurons were evaluated.

Physiological studies

Surgical Procedures

All procedures were carried out in accordance with the guidelines set by the US Society for Neuroscience and the National Institutes of Health and University of California, Irvine. The minimal possible number of cats (n=45) was used to obtain reproducible and statistically significant results. Cats (4-5 kg) of both sexes were pre-anesthetized with ketamine (40 mg/kg, sc) followed with intravenous injection of α-chloralose (50 mg/kg). Then a femoral vein and artery were cannulated for administration of drugs and fluids and measurement of arterial blood pressure (Statham P 23 ID, Oxnard, CA, USA). To maintain adequate depth of anesthesia, supplemental α-chloralose (5–10 mg/kg, i.v.) was given if the animals exhibited a corneal reflex, withdrew a limb in response to a
noxious stimulus during the experiment or displayed an unstable respiratory pattern or blood pressure. Heart rate was derived from the arterial blood pressure pulse by a biotech (Gould Instrument, Cleveland, OH, USA). Blood pressures and heart rates were recorded and analyzed offline with a computer and CED Spike 2 Windows software. Intubation of the trachea facilitated artificial respiration of room air enriched with oxygen (Harvard pump, model 662, Ealing, South Natick, MA, USA). Arterial blood gases were examined frequently (Radiometer, Model ABL-3, Westlake, OH, USA) and were maintained within the normal physiological range (PO$_2$, 100–150 mmHg; PCO$_2$, 35–40 mmHg; pH 7.35–7.45) by intravenous administration of 8% sodium bicarbonate or by adjusting the ventilator. Body temperature was maintained between 36 and 38 ºC with a heating pad and an external heat lamp.

The other femoral vein was cannulated to position the tip of the cannula close to the right ventricle for administration of PBG (14). To quantify cardiac vagal input to the NTS, a lateral thoracotomy on the right was performed between the fourth and fifth ribs. Ribs were cut to access the cardiac branch of the vagus nerve. To confirm isolation of the cardiac branch a bipolar flexible platinum electrode was placed around the nerve, held in place with polyvinylidimethylsiloxone dental impression material (Pentron, Wallington, CT), and transiently stimulated to demonstrate a decrease in heart rate. To allow quantification of neuronal activity in the NTS, the stimulating electrode was connected to an isolation unit and a stimulator (Grass, model S88). The thoracic wall was closed to prevent desiccation and heat loss. In other animals the cervical vagus was isolated and stimulated to elicit decreases in heart rate and afferent input to the NTS. A craniotomy was performed after animals were stabilized with a Kopf stereotaxic head frame to expose the dorsal surface of the medulla to access the NTS.

Microinjection probe consisting of a stainless steel guide tube with an outer diameter of 0.75 mm and an injection cannula with an inner diameter of 0.4 mm were inserted into the NTS to examine the inhibitory cardiovascular responses. To determine the site of the NTS sensitive to PBG, the probe was inserted at 1, 2 or 3 mm lateral to the obex or
midline according to coordinates taken from Berman’s atlas (2). Unilateral insertion of
the electrode allowed maintenance of a more optimal physiological condition compared
to bilateral electrode insertion. A one- or three-barrel glass pipette electrode was used
to evaluate neuronal activity or evaluate neuronal activity and iontophoresis the
antagonist. One barrel of the glass pipette electrode was filled either with saline or the
GABA_A receptor antagonist (gabazine). The other two barrels contained a platinum
recording electrode in either 0.5 M sodium acetate containing 2% Chicago sky blue
(Sigma Chemical, St. Louis, MO) or 4 M NaCl to balance the current. A one or three-
barrel glass pipette or microinjection electrode was positioned perpendicularly to the
dorsal surface of the medulla using visual approximation, 0 to 0.5 mm rostral to the
obex and advanced ventrally approximately 0.8 mm to reach the NTS. A stimulating
electrode was positioned at angle of 36 degrees to the dorsal surface of the medulla,
3.5 mm lateral to the midline and 0.5 mm rostral to the obex, and advanced to depth of
~4 mm to the nucleus ambiguus to evaluate antidromically projection of the NTS neuron
to the nucleus ambiguus. At end of experiment, the recording and microinjection sites
were marked with Chicago blue dye for later histological confirmation following
administration of drugs into the NTS.

Acupuncture needles were inserted to a depth of about 4 mm, bilaterally, at the
Neiguan-Jianshi acupoints (P5-6). Needles at these acupoints were located 2–3 cm
proximal to the flexor crease on the cat’s wrist and were separated by 5–7 mm. They
were connected to an isolation unit and stimulator (Grass, model S88) to deliver bipolar
stimuli at 2 Hz, 0.5 ms and 2-4 mA.

Methods of Blockade

The importance of the NTS in the PBG response was determined by microinjecting
kainic acid (KA, 1 mM, 50 nl) (41) into three sites in this region. The role of GABA_A
receptors in the NTS during EA were evaluated by microinjecting gabazine (SR-95331,
27 mM, 50 nl, Sigma Aldrich, St. Louise, MO) (43) at a time when the cardiovascular
effects of EA were still present. Iontophoresis (Neuro Phore BH-2 system, Medical
System, Greenvale, NY) of the saline control or gabazine into the NTS was applied for approximately 2 min following EA stimulation. The antagonist also was iontophoresed during repeated stimulation of the vagus nerve in the absence of EA. A current of 120-130 nA was used for iontophoresis.

Stimulating Methods

Repeated stimulation (every 10 min) of cardiopulmonary serotonin receptors with PBG (40 µg/ml/kg, iv) or the vagus nerve (2 Hz, 0.4-1 mA, 0.5 ms) respectively induced decreases in blood pressure and heart rate or increases in NTS neuronal activity. The median nerves beneath P5-6 acupoints were stimulated bilaterally with EA at a frequency of 2 Hz, an intensity of 2 to 4 mA using 0.5 ms pulses (46). Confirmation of median nerve stimulation was achieved by noting slight paw twitches. We applied 30 min of continuous low frequency, low intensity EA to simulate clinical use of this procedure (21, 25). To elicit a decrease in heart rate, the cardiac vagal branch was transiently stimulated with 0.4 mA, 10 Hz and 0.5 ms while the cervical vagus was stimulated with 0.7 to 1 mA, 10 Hz and 0.5 ms. To support anatomical studies on NTS-nucleus ambiguus projection, fifteen NTS neurons were activated antidromically from the nucleus ambiguus with 2 Hz, ~10 µA and 0.5 ms.

Extracellular NTS Recordings

Single-unit activity of NTS neurons was recorded with a platinum electrode inserted in a three-barrel pipette positioned in the NTS. Action potentials were amplified with a preamplifier (Neuroprobe Amplifier Model 1600, A-M Systems, Inc.) attached to a Nerve Traffic Analysis System 662C-3 (Bioengineering, College of Medicine, University of Iowa), then filtered (3-10 KHz) and monitored with an oscilloscope (Tektronix 2201). Action potentials, blood pressures and heart rates were digitized and analyzed online with a Pentium IV computer and a four-channel data acquisition system program (SHMU; Shanghai Medical College of Fudan University, China) that uses wave shape recognition algorithms to allow detection of similar wave shapes, heights and latencies of response (23, 24). Peristimulus time histograms were constructed for each neuron to
assess evoked responses to stimulation of the vagal or median nerves. The relationship between NTS neuronal activity and blood pressure was assessed by both time and frequency domain analyses using arterial pulse triggered averaging and coherence analysis (23, 44-46). Examination of the responses to baroreceptor afferent input with either nitroprusside (50 μg/kg) or phenylephrine (2.5 μg/kg) provided additional characterization of NTS neurons. Each NTS neuron studied that received convergent input from P5-6 (median nerves), vagal (afferent) nerves and baroreceptors and displayed cardiac rhythmicity was stimulated with EA for 30 min.

Experimental Protocols

Role of NTS in cardiopulmonary reflex

Reflex decreases in blood pressure and heart rate were elicited by intravenous injection of PBG (40 μg/ml/kg, iv) every 10 min. Kainic acid (1 mM, 50 nl) was microinjected unilaterally into the NTS 1, 2 or 3 mm lateral to the obex after observing two repeatable decreases in blood pressure and heart rate in four animals. The principal site in the NTS responsible processing the Bezold Jarisch reflex response was then used for all subsequent studies.

Effects of EA on PBG evoked reflexes

Maximal decreases in blood pressure and heart rate were evaluated as the difference between mean arterial blood pressure (MAP) and heart rate before application of PBG and the lowest hemodynamic values during induction of the Bezold Jarisch reflex. We first examined for consistency of hemodynamic responses to PBG injected every 10 min in a group of five animals. In eight other subjects after obtaining two consistent responses, eight additional PBG reflex responses were evaluated during and after 30 min of EA at P5-6. In addition as a control for receptor blockade studies, 50 nl of saline was microinjected unilaterally into the NTS following stimulation of EA in a subgroup of seven animals.
**Electrophysiological studies in NTS**

Neurons in the NTS were activated every 10 min by stimulating the cardiac or cervical vagus nerves. Peristimulus histograms were constructed with histogram bars representing evoked activity over and above the basal discharge rate. Each neuron was characterized by assessing input during brief (30 to 60 s) stimulation of median nerve at the P5-6 acupoints. We examined only the neurons that received input from baroreceptors, identified as neurons responsive to nitroprusside or phenylephrine. We also evaluated the NTS response to intravenous PBG. Each neuron displayed a cardiac rhythmicity as determined by arterial pulse triggered activity averaging over a period of 5 min through analysis of the time and frequency domain relationships between blood pressure and cellular activity (pulse triggered activity and coherence, respectively). In some cases, we examined antidromically evoked activity in NTS neurons to identify direct NTS-nucleus ambiguus projections. These neurons were evaluated for collision of spikes during median nerve afferent-evoked orthodromic activity and nucleus ambiguus evoked antidromic action potentials in fifteen subjects. NTS neurons that responded to stimulation of the nucleus ambiguus with a constant latency were further examined for evidence of faithful responses at 200 Hz and for a stable threshold of the all-or-none evoked activity. The refractory period and the critical time interval (latency plus refractory period) were determined during the collision of the orthodromic and antidromic spikes (23).
Consistency of evoked responses in the absence of EA first was established during repeated vagal stimulation at 10 min intervals in five neurons. Then to evaluate the influence of EA on NTS neurons, cardiac or cervical vagal afferent evoked activity was determined repeatedly (every 10 min) before, during and after 30 min of EA in seven animals. In a subgroup of five of these animals, saline control was iontophoresed at end of EA. The influence of GABA<sub>A</sub> receptor blockade was assessed by iontophoresing gabazine (-120 mA, 2 min) immediately following termination of EA in five other animals.

**Statistical Analysis**

Number of neurons was tabulated (%) to determine NTS-nucleus ambiguous projection in relation to GABA<sub>A</sub> receptor and/or VGLUT3. Data are presented as means±SEM. Evoked activity during stimulation of vagal or median nerves was measured as the increase in number of spikes above baseline. Reflex changes in MAP and heart rate are presented as bar histograms. Data were plotted and analyzed with the Kolmogorov-Smirnov test for normal data distribution and normalized when necessary with Sigma plot (Jandel Scientific). The increase in cellular activity and decreases in blood pressure and heart rate before and after delivery of experimental drugs or saline or application of EA were compared using a one-way ANOVA followed post hoc with the Student-Newman Keuls test. Additionally, the saline group was compared with gabazine treatment group using a two-way ANOVA. All statistical analyses were performed with Sigma Stat (Jandel Scientific). The P< 0.05 level was used to detect significant differences.

We also evaluated time and frequency relationships between NTS neuronal activity and arterial blood pressure using pulse-triggered averaging as well as coherence analysis, as we have described previously (23, 24). Time domain analyses involved ECG or arterial pulse-triggered averaging. A threshold was set at the peak of the ECG wave or the systolic phase of the arterial pulse while another threshold was used for spike height discrimination and waveform recognition to sort action potentials during the 300 s evaluation period. Averages of the arterial pulse and histograms of NTS neuronal
activity were constructed (27, 28). Frequency domain analysis involved assessment of the coherence between NTS activity and arterial blood pressure using a Fast Fourier Transform (FFT) algorithm. We recorded data using a sampling rate of 10,000 Hz. Reconstructed data utilized every tenth sample, including assessment of the mean and peak amplitudes and the maximum and minimum slopes of the original spike to preserve the action potentials. The spikes were sorted and identified with a window discriminator to construct histograms prior to coherence analysis. The number of data sections (15-20 each lasting for 12.8 s) was chosen to determine the average histogram. Autospectra of NTS discharge and arterial blood pressure were generated with a FFT algorithm. Thus, coherence was generated with seven overlapping windows, each with a length of 12.8 s, consisting of 256 bins, with bin widths of 50 ms. The auto-spectral analysis was adopted from Shin et al., 1995 (34) using contiguous segments of 256 beats with 50% overlap between the segments. The frequency resolution was 1/12 s or 0.08 Hz. The coherence function (normalized cross-spectrum) provided a measure of the strength of linear correlation of NTS neuronal activity and blood pressure at each frequency. Coherence values of $\geq 0.5$ were chosen to reflect a statistically significant relationship between NTS spikes and arterial blood pressure (46).

Histology

At the end of each experiment, animals were euthanized under deep $\alpha$-chloralose anesthesia followed by injection of saturated KCl. Recording and/or microinjection sites were marked by either iontophoresis and/or microinjection of 2% Chicago blue dye. Thereafter, the brain was removed and fixed in 10% paraformaldehyde for at least 2 days. Brain stems were sliced with a microtome cryostat in 60-µm coronal sections. Recording and microinjection sites were reconstructed from the dye spots with the aid of a microscope (Nikon) and software (Corel presentation). The sites were plotted at to 0.6 mm rostral to the obex (2).
Results

Anatomical studies

Two animals were eliminated from this study since the sites for microinjection were found to be outside the nucleus ambiguus. Thus, three rats in which the microinjection site of the retrograde tracer was found inside the nucleus ambiguus were included in this study. The location of injected tracer (100 nl) in the medulla closely matched the coordinates of the nucleus ambiguus as defined by Paxinos and Watson's atlas for the rat (30). This microinjection site was within 1.6–2.2 mm lateral from the midline and 1.0–1.6 mm ventral to surface of the medulla and was observed ventrally adjacent to the rostral-ventral-respiratory-group. The tracer did not spread to caudal-ventrolateral-reticular-nucleus, containing C1 adrenaline cells and/or A1 noradrenaline cells (30). The distributions of the tracer in dorso-ventral planes ranged approximately from 0.10 x 0.26 mm to 0.33 x 0.52 mm and at rostral-caudal extension from 0.48 to 0.56 mm (Fig. 1, top panels).

We consistently observed that neurons labeled with the retrograde microsphere tracer were distributed rostrally and caudally throughout the NTS when the tracer was deposited in the nucleus ambiguus of the three rats. The labeled neurons were located in commissural, medial, ventral and lateral subdivisions of NTS in the rat, mainly at levels from Bregma -12.6 to -15.0 mm (30). Approximately two-thirds of the neurons labeled with microspheres in the NTS were found to be located ipsilateral to the injection site in the nucleus ambiguus.

In all three rats, VGLUT3, GAD67 and GABA_A receptors were distributed bilaterally throughout the caudal and rostral NTS. Cell bodies were stained with tracer, VGLUT3 or GABA_A receptors while neuronal processes were labeled with GAD67.

We evaluated the relationship between VGLUT3 + tracer-labeled NTS neurons and GABA_A receptors. More than half of retrograde tracer-labeled NTS neurons were double-labeled with either VGLUT3 or GABA_A receptors (Table 1). The majority of neurons double-stained with the retrograde tracer + VGLUT3 (about 71%) also were labeled with GABA_A receptors (Table 1 and Fig. 1, bottom). Neural processes labeled...
with GAD67 were in close apposition to the majority of NTS neurons that contained the retrograde tracer alone as well as neurons containing both the retrograde tracer and VGLUT3 (Fig. 2).

**Physiological studies**

**Role of NTS in cardiopulmonary reflex**

Kainic acid microinjected 0-0.5 mm rostral to the obex and between 1.6 to 2.2 mm lateral to midline (intermediate NTS) reduced the Bezold Jarisch responses by 12 mmHg and 16 beats/min (Fig. 3). In contrast, kainic acid microinjected 0.5 to 1.0 mm and 3 to 3.4 mm lateral to the midline did not alter the cardiopulmonary reflex responses. Blockade with KA did not influence baseline blood pressure and heart rate. The PBG-induced blood pressure and heart rate responses were not affected by KA microinjection ventral to the NTS.

**Effects of EA on PBG evoked reflexes**

We observed consistent decreases in blood pressure and heart rate with repeated stimulation of cardiopulmonary serotonin receptors with PBG every 10 min (Fig. 4A). The baseline blood pressures and heart rates before each Bezold Jarisch reflex were not significantly different throughout the experiment. The inhibitory cardiovascular responses were reduced by EA applied at the P5-6 acupoints for 30 min. The modulatory effect of EA on the PBG-induced decrease in blood pressure lasted for 80 min while the bradycardia persisted for 70 min (Fig. 4B). EA did not influence baseline blood pressure or heart rate (42). Gallamine triethiodide, used to inhibit muscle movement during stimulation of median nerve with EA at P5-6 as in previous studies did not influence the response to EA (42).

**NTS GABA mechanisms in EA cardiopulmonary reflex modulation**

EA modulation of the PBG evoked bradycardia responses was reversed for at least 10 min by GABA\textsubscript{A} receptor blockade in intermediate NTS (compare Fig. 4B and Fig. 5).
The acupuncture effect outlasted the action of gabazine for at least 10 min. Gabazine did not influence the action of EA on the blood pressure response (Fig 5).

Microinjection of gabazine into the NTS transiently increased baseline heart rate from 206±9 to 211±9 beats/min while baseline blood pressure tended to increase from 123±7 to 129±5 mmHg in nine subjects. Baseline heart rate and blood pressure were restored to normal levels before the next administration of PBG. Blockade of GABA_A receptors in the NTS did not influence the Bezold Jarisch reflex blood pressure (-16±4 vs. -14±3 mmHg) or heart rate response (-20±7 vs. -22±10 beats/min) in four subjects.

**NTS neuronal activity during EA**

Before evaluation of their responses to repeated activation of cardiac vagal afferents and superimposition of EA, NTS neurons were characterized using time and frequency domain analysis. Baseline discharge activity of the NTS neurons was 3.8±0.6 spikes/s. Forty five neurons displayed cardiac rhythmicity (Fig. 6A and B) while a subset of 17 cells also were shown to integrate baroreceptor input (Fig. 6C) and receive convergence from vagus and median nerve afferents. All 17 NTS neurons also were activated following PBG administration (Fig. 6D). Two of 15 neurons responsive to EA projected directly to the nucleus ambiguus since they could be driven antidromically from the nucleus ambiguus, were faithful to high frequency stimulation and displayed a constant latency (Fig. 6E).

In the absence of EA, five NTS cells examined for repeated stimulation of cardiac vagal afferents induced consistent activity (Fig. 7A). However, 30 min of EA stimulation at P5-6 reduced NTS vagally evoked activity for at least 60 min in seven neurons (Fig. 7B). Modulation by EA was reversed by blockade of GABA_A receptor (n=5) (Fig. 7C).

**Histology**
The microinjection and recording sites in the NTS were confirmed histologically to be 1.6 - 2.2 mm lateral to the midline and 0.7 - 1.4 mm from the surface and at 0.6 to 0 mm rostral to the obex (Fig. 7) as shown in Berman’s atlas (2). The sites were determined by the locations of microinjection tracks and dye spots.

Discussion

EA applied at the P5-6 acupoints inhibits sympathoexcitatory activity and reflex responses through long-loop neuronal pathways that involve the hypothalamic arcuate nucleus, midbrain ventrolateral periaqueductal gray, medullary raphé pallidus and rostral ventrolateral medulla. The present study extends these observations by demonstrating that EA at the same acupoints modulates parasympathoexcitatory (inhibitory cardiovascular) responses in the NTS that projects to the nucleus ambiguus to regulate heart rate. In this regard, prolonged activation of sensory fibers in the median nerves for 30 min using low frequency and low voltage modulates PBG-related bradycardia responses through a GABAergic system in the NTS. Combined physiological and immunohistochemical studies demonstrate that GABA contributes to the EA-related modulatory effect on the negative chronotropic response through activation of GABA_{A} receptors in NTS neurons that ultimately project to the nucleus ambiguus.

We have used rats rather than cats to demonstrate the presence of nucleus ambiguus projecting NTS neurons that are juxtaposed to GABAergic fibers and expressing GABA receptors. Our laboratory has shown that the anatomic circuitry in the cardiovascular responses in rats and cats are virtually identical (9, 23, 28, 38, 42). Therefore, we believe that our data obtained in rats likely also apply to cats.

We anatomically showed the presence of NTS-nucleus ambiguus projections with microinjection of microsphere tracer in the nucleus ambiguus. Although unlikely, the
tracer potentially could stain both cardiovascular and respiratory neurons. To determine if nucleus ambiguus projecting NTS neurons participate in the PBG evoked and cardiovascular responses, we further examined the projections with extracellular recording and antidromic stimulation. We showed that NTS-nucleus ambiguus neurons displayed cardiac rhythmicity, and were responsive to baroreceptor stimulation and PBG application. Thus, while the retrograde tracing provided anatomical evidence for a direct projection, we functionally supported this finding with characterization of cardiovascular NTS-nucleus ambiguus neuronal projection displaying cardiac rhythmicity and neuronal responses to PBG.

Vesicular glutamate transporters (VGLUTs) transport specifically glutamate into vesicles of neurons, and thus offer a unique marker to distinctively identify neurons that use glutamate as a neurotransmitter (12). Although there has been discussion about the specificity of VGLUT3 for glutamatergic neurons, the VGLUT3 labeling represents a valuable way to visualize glutamatergic neurons in the brain (12, 15, 16, 33). VGLUT3 labeled NTS neurons projecting to nucleus ambiguus are co-localized with GABA\textsubscript{A} receptors or juxtaposed to GABAergic nerve fibers. The apposition of the two neuronal structures suggests that GABAergic fibers may synapse on a glutamatergic NTS-nucleus ambiguus neuron. Although confocal microscopic images display juxtaposed positioning, it is unclear if synaptic transmission occurs between the neuronal processes. In this regard, our physiological data demonstrated activation of GABA\textsubscript{A} receptors in cardiovascular PGB-responsive NTS neurons in support of a GABA synaptic transmission. Thus, the current findings show the potential for GABA to influence glutamatergic NTS-nucleus ambiguus projections.

Neuroanatomical and electrophysiological studies have shown that neurons in the NTS process sensory input from vagal afferents with endings originating from the gastrointestinal tract, baroreceptors and cardiopulmonary region. Injection of PBG into the right atrium activates cardiopulmonary vagal afferent C fibers (7, 8, 29) and the NTS (18). The present experiments demonstrate that cardiovascular NTS neurons activated
by PBG and vagal afferent stimulation are influenced by somatic afferent input. Thus, NTS vagal-evoked activity is reduced by 30 min of median nerve stimulation (EA). Moreover, the vagal-evoked NTS neurons are inhibited by EA for a prolonged period of time during and following 30 min of median nerve stimulation, a hallmark of EA. Thus, the NTS processes convergent input from both visceral and somatic afferent fibers and their interaction influences cardiopulmonary-related vagal reflex bradycardia.

In the present study, we demonstrated that GABA in the NTS contributes importantly to EA modulation of the profound bradycardia consequent to activation of cardiopulmonary serotonin receptors. Blockade of GABA<sub>A</sub> receptors in the NTS transiently increased baseline heart rate confirming that GABA tonically inhibits premotor parasympathoexcitatory NTS neurons (32). However, baseline heart rates and blood pressures returned to preblockade levels before subsequent administration of PBG, suggesting that the responses to gabazine were not influenced by the transient baseline changes. EA modulation of NTS evoked activity through a GABA<sub>A</sub> mechanism is similar to the prolonged actions by EA in the nucleus ambiguus and the rostral ventrolateral medulla (38, 42).

The NTS processes sympathetic and parasympathetic activity differentially and possibly controls these pathways independently (31, 55). We have observed that kainic acid in the NTS partially reduces both the bradycardia and depressor responses while action of EA through GABA in NTS partially influences heart rate but not blood pressure changes. A similar discrepancy has been reported in the nucleus ambiguus (42) demonstrating that both the NTS and the nucleus ambiguus regulate EA modulatory action on heart rate. Moreover, the NTS processes the initial cardiopulmonary reflex inhibitory responses and projects to the caudal and rostral VLM (47) that could regulate vasomotor tone and the effects of EA on inhibitory hemodynamic responses (38). Thus, the restoration of blood pressure with EA during the Bezold Jarisch reflex likely occurs in vasomotor centers such as the rVLM (28, 46) suggesting unique roles of various
brain stem nuclei in EA mechanisms of regulation of reflex lowering of heart rate and blood pressure.

Electroacupuncture appears to be capable of normalizing blood pressure by lowering increased blood pressure and elevating decreased blood pressure. The somatic sensory nerve evoked input during acupuncture through specific neurotransmitters decreases the extent of neuronal excitation associated with increased sympathetic outflow and lowers elevated blood pressure (25, 46). On the other hand, if acupuncture is applied in the presence of reflex sympathetic withdrawal and/or increased parasympathetic outflow, the somatic sensory input activates modulatory neurotransmitter systems to reduce the extent of hypotension and bradycardia (38, 42). Thus, acupuncture normalizes blood pressure by modulating elevated neuronal activity in various brain stem regions known to be important in regulating autonomic function, including both sympathetic and parasympathetic nerves.

Perspectives and Significance

Acupuncture can profoundly influence cardiovascular function and may serve a role in medical conditions associated with low blood pressure. This condition occurs, for example, during hemorrhage. Syuu et al. reported that EA reverses hemorrhage-induced hypotension, possibly by increasing venous return through enhanced vasomotor tone and the preload (36). In a more clinically related study, acupuncture has been shown to elevate blood pressure in patients with shock (5). With the exception of our previous study that acupuncture modulates cardiopulmonary inhibitory heart rate responses through GABAergic inhibition of parasympathetic preganglionic neurons in nucleus ambiguus (42), the underlying central neural mechanisms associated with action of EA in hypotension have not been evaluated previously.

Activation of cardiopulmonary vagal afferents to lower heart rate shares many features of vasovagal syncope. Vasovagal syncope is the most common cause of transient
unconsciousness (50) and is thought to be mediated by activation of a cardiopulmonary mediated reflex (1). EA may serve as a therapeutic option for subjects at risk for recurrent vasovagal syncope.

Conclusion

Through GABA<sub>A</sub> receptor mechanism 30 min of electroacupuncture reduces augmented activity of parasympathetic premotor NTS neurons and reverses heart rate during PBG stimulation of the cardiopulmonary afferents.

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Disclosures

None are declared by the authors.
Table 1. Neurons labeled with the retrograde tracer, VGLUT3 and/or GABA\textsubscript{A} receptors in the NTS of rats.

<table>
<thead>
<tr>
<th>Labeling</th>
<th>((\text{Bregma -13.56 mm})) (n=3)</th>
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<tbody>
<tr>
<td>Tracer (#)</td>
<td>138 ± 24</td>
</tr>
<tr>
<td>VGLUT3 (#)</td>
<td>179 ± 19</td>
</tr>
<tr>
<td>GABA\textsubscript{A} (#)</td>
<td>97 ± 12</td>
</tr>
<tr>
<td>Tracer + VGLUT3 (#)</td>
<td>80 ± 20</td>
</tr>
<tr>
<td>Tracer + GABA\textsubscript{A} (#)</td>
<td>70 ± 11</td>
</tr>
<tr>
<td>Tracer + VGLUT3 + GABA\textsubscript{A} (#)</td>
<td>57 ± 13</td>
</tr>
<tr>
<td>(Tracer + VGLUT3)/Tracer (%)</td>
<td>58 ± 5</td>
</tr>
<tr>
<td>(Tracer + GABA\textsubscript{A})/Tracer (%)</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>(Tracer + VGLUT3 + GABA\textsubscript{A})/(Tracer + VGLUT3) (%)</td>
<td>71 ± 4</td>
</tr>
</tbody>
</table>

Means ± SD:

Average number of neurons labeled with retrograde microsphere tracer (Tracer) injected into nucleus ambiguus, VGLUT3 and/or GABA\textsubscript{A} receptors in the nucleus of tractus solitarius (NTS). Percentages of neurons (%) stained with tracer and labeled with VGLUT3 or GABA\textsubscript{A} relative to Tracer.

Percentages of neurons (%) stained with tracer and labeled with VGLUT3 and GABA\textsubscript{A} relative to Tracer and VGLUT3.
Figure legends

Figure 1. Top: Section of rat medulla oblongata illustrating microinjection sites of microsphere tracer. Panel A: Illustrates a section of rat medulla (Bregma -13.32 mm) according to Paxinos and Watson’s rat brain atlas. The red spot indicates injection site of retrograde tracer. Panel B: Displays image of an original brain section that matches the plane shown in the panel A. The bright red area indicated by the arrow demonstrates the injection site of rhodamine-labeled fluorescent microspheres into the nucleus ambiguus (NAmb). RVRG, rostral-ventral-respiratory-group; CVL, caudal-ventrolateral-reticular-nucleus. Scale bars in Panels A and B represent 1 mm. Bottom: Confocal microscopic images demonstrate triple-fluorescent labeling in the NTS (Bregma -13.56 mm) of a rat. Arrows in Panels A, B, C, and D indicate neurons stained with VGLUT3, GABA\(_A\) receptors, retrograde tracer originating from nucleus ambiguus, and co-labeling of VGLUT3, GABA\(_A\) and tracer, respectively. Scale bars represent 20 \(\mu\)m.

Figure 2. Confocal microscopic images showing triple-fluorescent labeling in the NTS (Bregma -13.56 mm) of a rat. Arrows in Panels A, C and D indicate neurons containing VGLUT3, the retrograde tracer originating from nucleus ambiguus, and co-labeling of VGLUT3 and tracer, respectively. Arrowheads in Panels B and D indicate neural processes containing GAD67. In Panel D, the neural processes labeled with GAD67 (green) are in close proximity to the perikarya stained with VGLUT3 and the retrograde tracer indicated with an arrow. Scale bars represent 20 \(\mu\)m.

Figure 3. Bar histograms display decreases in mean blood pressure (ΔMAP) and heart rate (ΔHR) to PBG before and after microinjection of kainic acid (KA) in the intermediate NTS. KA transiently reduced parasympathoexcitatory hemodynamic and negative chronotropic reflex responses. Baseline blood pressure and heart rate are shown above each bar as means and SEM. *, Indicates significant difference compared to control PBG responses.
Figure 4. Decreases in MAP and HR were reduced with electroacupuncture (EA). Bar histograms display consistent responses to repeated PBG (every 10 min). EA reduced the decrease in MAP and HR for at least 70 min. Microinjection of saline into the NTS did no influence the inhibitory cardiovascular reflex responses. Baseline blood pressure and heart rate are shown above each bar as means±SEM. *, Indicates significant difference compared to control PBG responses.

Figure 5. EA modulation of the PBG-induced bradycardia (right panel) but not hypotension (left panel) is transiently reversed by gabazine. * indicates significant difference compared to baseline PBG responses, while † shows significant difference from preceding EA PBG-EA response. *, Below bar h indicates end of gabazine’s activity and the effect of EA. Letters in a–h shown in the bars correspond to the original tracings above showing decreases in MAP and HR. Baseline MAP and HR are shown above bars as means±SEM.

Figure 6. Characterization of NTS neurons. Panels A and B respectively display time and frequency (coherence of 0.87 and frequency 2.4) domain analyses to show cardiac rhythmicity. Panel C shows decreased NTS activity during a nitroprusside-evoked decrease in BP, demonstrating that it was barosensitive. The NTS neuron also increased its discharge rate in response to iv PBG (Panel D, closed arrow indicates time of NTS activity shown with neurogram). Antidromic stimulations show that the neuron projects to the nucleus ambiguus (Panel E). The NTS neuron activated antidromically by 2 Hz, 11 μA and 0.5 ms nucleus ambiguus (NAm) stimulation (↓) collided with the orthodromically median nerve (MN, P5-6, *) evoked spike (Panel E, middle tracing). # indicates the antidromic spike that is absent during collision, middle panel. Critical interval was 13.5 ms and refractory period was 4.86 ms. PSD, power spectral density.

Figure 7. Bar histograms display NTS neuronal evoked activity during repeated stimulation of vagal afferents every 10 min. Panel A shows consistent evoked activity with stimulation of vagal afferents. Panels B and C show that EA reduced evoked
activity for at least 60 min through GABA mechanism. Blockade with gabazine (Panel C) reversed the effect of EA (c) compared to pre-blockade (b) in contrast to saline (Panel B). Letters a-c in bars correspond with peristimulus histograms (Panel C).

**Figure 8.** Composite map displays the sites of microinjections, iontophoresis and extracellular recordings in the intermediate NTS of cats. For ease of representation, all sites are displayed on the right and microinjections on the left. Microinjections with KA at 1 and 3 mm lateral to midline also are shown on the left NTS. *, sites located within NTS. ○, control site outside intermediate NTS. Coronal section is 0 to 0.6 mm rostral to obex.


15. **Gritti I, Henny P, Galloni F, Mainville L, Mariotti M and Jones BE.** Stereological estimates of the basal forebrain cell population in the rat, including neurons containing choline acetyltransferase, glutamic acid decarboxylase or phosphate-activated glutaminase and colocalizing vesicular glutamate transporters. *Neuroscience* 143: 1051-1064, 2006.


Fig 1
Fig 5

MAP (mmHg)

-80  -60  -40  -20  0

n=5  Gabazine (NTS)

EA

HR (beats/min)

-100  -80  -60  -40  -20  0

n=5  Gabazine (NTS)

ΔMAP (mmHg)

-60  -40  -20  0

n=5

ΔHR (beats/min)

-80  -60  -40  0

n=5
Fig 6