Modulation of Cardiopulmonary Depressor Reflex in Nucleus Ambiguus by Electroacupuncture: Roles of Opioids and Gamma Aminobutyric Acid

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Abstract

Stimulation of cardiopulmonary receptors with phenylbiguanide (PBG) elicits depressor cardiovascular reflex responses, including decreases in blood pressure and heart rate mediated by the brainstem parasympathetic cardiac neurons in the nucleus ambiguus (NAmb). The present study examined NAmb neurotransmitter mechanisms underlying the influence of electroacupuncture (EA) on the PBG-induced hypotension and bradycardia. We hypothesized that somatic stimulation during EA modulates PBG responses through opioid and γ-aminobutyric acid (GABA) modulation in the NAmb. Anesthetized and ventilated cats were studied during repeated stimulation with PBG or cardiac vagal afferents while low frequency EA (2 Hz) was applied at P5-6 acupoints overlying the median nerve for 30 min and NAmb neuronal activity, heart rate and blood pressure were recorded. Microinjection of kainic acid into the NAmb attenuated the PBG-induced bradycardia from -60±11 to -36±11 beats/min. Likewise, EA reduced the PBG-induced depressor and bradycardia reflex by 52 and 61%, respectively. Cardiac vagal afferent evoked preganglionic cellular activity in the NAmb was reduced by EA for about 60 min. Blockade of opioid or GABA\textsubscript{A} receptors using naloxone and gabazine reversed the EA-related modulation of the evoked cardiac vagal activity by 73 and 53%, respectively. Similarly, naloxone and gabazine reversed EA modulation of the negative chronotropic responses from -11±5 to -23±6 and -13±4 to -24±3 beats/min, respectively. Thus, EA at P5-6 decreases PBG evoked hypotension and bradycardia as well as the NAmb PBG sensitive preganglionic cardiac vagal outflow through opioid and GABA neurotransmitter systems.
Introduction

Cardiovascular and autonomic nervous system abnormalities affecting blood pressure, including elevations in blood pressure, can be improved with electroacupuncture (EA). (10; 21) Additionally, reflex increases in sympathetic activity are reduced by EA in both patients and experimental studies. (20; 34) Thus, sympathoexcitatory-induced increases in blood pressure are reduced by EA at P5-6, which overlie the median nerves. (35) We have shown in a series of studies that several neurotransmitter systems, including opioids and γ-aminobutyric acid (GABA) participate in the inhibitory influence of acupuncture. (11; 12; 33; 39) The rostral ventrolateral medulla (rVLM) and nucleus râphe pallidus (NRP) in the brainstem, which regulate sympathetic outflow, receive input from P5-6, two acupoints that exert strong cardiovascular actions. (24; 28) Thus, EA reduces premotor sympathoexcitatory cardiovascular rVLM neuronal activity which, in turn, decreases reflex elevations in blood pressure. (34; 36) Despite this large body of evidence showing that EA regulates blood pressure through its action on the sympathetic nervous system, there is less information about its action on the parasympathetic system. (42; 43)

EA can modulate decreases in blood pressure. In this regard, acupuncture appears to be able to partially reverse experimental hemorrhagic hypotension (32) and nitroprusside induced hypotension (47). Preliminary studies also suggest that EA can reduce gastric distention-induced hypotensive reflex responses. (14) The mechanisms by which EA influences central processing to reverse the hypotensive responses are unclear.

Activation of cardiopulmonary unmyelinated chemosensitive vagal afferents by prostaglandin-E2, veratrum alkaloids, serotonin, capsaicin or intravenous phenylbiguanide (PBG) reflexly inhibits heart rate and blood pressure. (7; 8; 15) This phenomenon, originally described and characterized by Bezold, Hirt, Richter and Jarisch, is known as the Bezold-Jarisch reflex. (18)

Recently, several studies have examined brain stem regions that process cardiovascular responses during the Bezold-Jarisch reflex. These include a number of medullary regions, such as the rVLM, cVLM, and NTS (3; 15; 41). The negative chronotropic response during PBG stimulation results from excitation of cardiac parasympathetic neurons processed, in part, through the nucleus ambiguus (NAmb). (27; 44)
Opioids and GABA are present in the NAmb and influence heart rate. GABA functions as an inhibitory neurotransmitter to increase heart rate. Enkephalin microinjected into the NAmb in contrast decreases heart rate. Additionally, we recently have shown that cholinergic preganglionic parasympathetic neurons in the NAmb activated by EA are situated in close proximity to fibers containing enkephalin, suggesting the potential for interaction. These studies suggest that modulation of NAmb neuronal activity by EA may reduce PBG-induced bradycardia and cardiac vagal evoked activity in the NAmb, possibly through an opioid or a GABAergic mechanism. In the present study, we therefore hypothesized that EA is capable of reducing the bradycardia and hypotension evoked by stimulation of the Bezold-Jarisch reflex through its action on neural processing in the NAmb. We further hypothesized that opioids and GABA in the NAmb participate in EA modulation of the PBG-related cardiovascular inhibition.

Materials and Methods

Surgical Procedures

The animal use and care committee at the University of California, Irvine, approved all surgical and experimental protocols of this study. All procedures were carried out in accordance with the US Society for Neuroscience and the National Institutes of Health guidelines. The minimal possible number of cats was used to obtain reproducible and statistically significant results. Cats of both sexes were preanesthetized with ketamine (40 mg/kg, sc). A femoral vein and artery then were cannulated for administration of drugs and fluids and measurement of arterial blood pressure (Statham P 23 ID, Oxnard, CA, USA). Subsequently, α-chloralose (50 mg/kg) was administered intravenously. 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 (HR) was derived from the arterial blood pressure pulse by a biotech (Gould Instrument, Cleveland, OH, USA). Blood pressures and HRs were recorded and analyzed offline with a Pentium IV 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$_2$, 28–35 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 °C with a heating pad and an external heat lamp.

The femoral vein was cannulated to position the tip close to the right ventricle for delivery of PBG. A lateral thoracotomy was performed between the right 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 and transiently stimulated to elicit a decrease in heart rate. To quantify neuronal activity in the NAmb, the stimulating electrode was connected to an isolation unit and a stimulator (Grass, model S88) and was held in place with hypoxy dental glue (Pentron, Wallington, CT). The thoracic wall was closed to prevent desiccation and heat loss. A craniotomy was performed after the animal was stabilized with a Kopf stereotaxic head frame to expose the dorsal surface of the medulla to access the NAmb.

Microinjection electrodes consisting of a 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 NAmb to examine the inhibitory cardiovascular responses. The electrode was connected with fluid filled tubing to a 1 µl Hamilton syringe that we used to deliver a volume of 50 nl. A three-barrel glass pipette electrode was used to evaluate neuronal activity and iontophoretic antagonists. One barrel of the glass pipette electrode was filled with saline or the GABA$_A$ receptor antagonist (gabazine). The other two barrels contained a platinum recording electrode with 0.5 M sodium acetate containing 2% Chicago sky blue (Sigma Chemical, St Louis, MO) and 3 M NaCl to balance the current. Using coordinates taken from Berman’s atlas(5), a three-barrel glass pipette or microinjection electrode was positioned perpendicularly to the dorsal surface of the medulla, 0.5 mm rostral and 3.5 mm lateral to obex, and advanced ventrally approximately 3.7 mm to reach the NAmb. Insertion of an electrode within the NAmb was confirmed with microinjection of 50 nl DLH (4 nM), which typically decreased heart rate ~15 bpm. We found that unilateral insertion of the electrode allowed maintenance of a physiologically more optimal condition than with bilateral electrode insertion. 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
NAmb. Acupuncture needles were inserted bilaterally to a depth of about 4 mm 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.

**Methods of Blockade**

The importance of the NAmb in the PBG response was determined by microinjecting kainic acid (KA, 1 mM, 50 nl)(36) into this region. The roles of μ-opioid and GABA_A receptors in the NAmb during EA were evaluated by unilateral microinjection of naloxone (100 nM, 50 nl, Sigma Aldrich, St. Louise, MO)(33) or gabazine (SR-95331, 27 mM, 50 nl, Sigma Aldrich, St. Louise, MO)(39) soon after terminating EA stimulation, at a time when the cardiovascular effects of EA were still present (as documented in control studies). Several of our previous studies have demonstrated significant blockade with unilateral administration of naloxone or gabazine, allowing demonstration that the nucleus plays a role in the EA-cardiovascular response.(33; 34; 39) Saline served as the control. Iontophoresis (Neuro Phore BH-2 system, Medical System, Greenvale, NY) of gabazine or naloxone into the NAmb was applied following EA stimulation for approximately 2 min. The antagonists also were iontophoresed during repeated stimulation of the vagal nerve in the absence of EA. Gabazine (27 mM) or naloxone (100 nM) was iontophoresed at a current of 120-130 nA, as in our previous studies and in investigations by others. (16; 33)

**Stimulating Methods**

Repeated stimulation (occurring every 10 min) of cardiopulmonary serotonin receptors with PBG (40 µg/ml/kg) to evoke the Bezold-Jarisch reflex or electrical stimulation of afferents in the cardiac vagal nerve (2 Hz, 0.4-0.6 mA, 0.5 ms) during electrophysiological recordings were used to induce consistent decreases in blood pressure and increases in neuronal NAmb activity, respectively. Gallamine triethiodide (4 mg/kg) was administered intravenously before application of EA or recording neuronal activity to avoid muscle movement during stimulation of somatic nerves. The median nerves beneath P5-6 acupoints were stimulated bilaterally
during EA at a frequency of 2 to 4 Hz, an intensity of 2 to 4 mA using 0.5 ms pulses.(33) We applied 30 min of continuous EA to simulate clinical use of this procedure. Additionally, to determine if neurons in the NAmb were preganglionic vagal neurons, the cardiac vagal branch was stimulated (0.1-0.4 mA, 2 Hz and 0.5 ms) using antidromic collision techniques.

**Extracellular NAmb Recordings**

Single-unit activity of NAmb neurons was recorded with a platinum electrode inserted into a three-barrel pipette positioned in the NAmb. Action potentials were amplified with a preamplifier (Grass P511), attached to a high-impedance probe (Grass H1P5), then filtered (0.3-10 kHz) and monitored with an oscilloscope (Tektronix 2201). Data were analyzed offline with a Pentium IV computer and CED Spike 2 Windows software. Action potentials were analyzed both visually and with a SHMU 2006 (Shanghai Medical College of Fudan University, China) program using wave shape recognition algorithms to allow detection of similar wave shapes, heights and latencies of response.(23; 25) Peristimulus time histograms were constructed for each neuron to assess evoked responses to stimulation of the cardiac vagal or median nerves. The relationship between NAmb neuronal activity and blood pressure was assessed by both time and frequency domain analyses using arterial pulse triggered averaging and coherence analysis.(25; 33-35) Examination of the responses to baroreceptor afferent input with either nitroglycerin (2.5 mg/ml) or phenylephrine (2 mg/ml) provided additional characterization of NAmb neurons. Each NAmb neuron studied that received convergent input from P5-6 (median nerves), cardiac vagal (afferent) nerves and baroreceptors and displayed cardiac rhythmicity was stimulated with EA for 30 min.

**Experimental Protocols**

*Effects of EA on PBG evoked reflexes*

To decrease repeatedly blood pressure and heart rate, cardiopulmonary afferents were activated with intravenous injections of PBG every 10 min. 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 MAP and heart rate during reflex stimulation. We first examined for consistency of hemodynamic responses to PBG in a group of five animals. In six other subjects after obtaining two repeatable
responses, eight additional PBG reflex responses were evaluated during and after 30 min of EA. As a control, for receptor blockade studies, saline (50 nl) was microinjected into the NAmb after the end of EA.

**Role of NAmb in cardiopulmonary reflex**

Reflex decreases in blood pressure and heart rate were induced with intravenous injection of PBG every 10 min. Kainic acid was microinjected after two repeatable decreases in blood pressure and heart rate in six animals.

**NAmb opioid and GABA systems in EA cardiopulmonary reflex modulation**

Opioid and GABA\(_A\) receptors in the NAmb were blocked to evaluate their role in mediating the action of EA on hypotension and bradycardia. Similar to protocols used to evaluate influence of EA on PBG-evoked inhibitory reflex responses, either naloxone or gabazine was microinjected into the NAmb of twelve animals (six in each group).

**Electrophysiological studies in NAmb**

Neurons in the NAmb were activated every 10 min by stimulating the cardiac vagal nerve. Peristimulus histograms were constructed with bars representing evoked activity over and above the basal discharge rate. Each neuron was characterized by assessing input during median nerve stimulation at acupoints P5-6. We selected only cells that received input from baroreceptors tested by administration of nitroglycerin or phenylephrine. Each neuron displayed a cardiac rhythmicity determined by arterial pulse triggered 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 NAmb neurons, using a frequency of 2 Hz and pulse duration of 0.5 ms, to identify neurons that functioned as preganglionic parasympathetic cells. Stimulation of the cardiac vagus nerve activated NAmb neurons orthodromically and antidromically. The orthodromically activated neurons showed variable latencies. In contrast, the antidromically activated NAmb neurons responded to the stimulation with a constant latency. Neurons that responded to antidromic stimulation were examined further for a constant latency with repeated stimulation, a stable threshold of the evoked all-or-none response and a faithful response to high rates of stimulation (200 Hz).
Regular responses to high frequency stimulation helped establish an absence of variable synaptic delay. Then, the neurons were evaluated for collision of cardiac vagal afferent evoked antidromic action potentials and median nerve-evoked orthodromic activity. The refractory period was measured to determine the critical time interval (latency plus refractory period) during which the ortho- and antidromic spikes can collide. The conduction velocity of preganglionic neurons was determined by dividing the distance between the recording and stimulating electrodes and the antidromic latency. Consistency of responses of five neurons was evaluated during repeated stimulation. In eight other subjects, after two consistent preganglionic neuron responses, 30 min of EA was applied followed by eight additional cardiac vagal stimulations.

**NAmb neuronal response to PBG and EA**

Cardiovascular NAmb neurons were examined for responsiveness to PBG. Four neurons were activated repeatedly by stimulation of cardiac vagal afferents before, during and after EA stimulation at P5-P6. The neurons also were responsive to baroreceptor stimuli.

**NAmb opioid and GABA systems in EA modulation of neuronal activity**

Cardiac vagal afferent evoked activity in the NAmb was evaluated repeatedly (every 10 min) before, during and after 30 min of EA. The influence of opioid or GABA_A receptor blockade was assessed by iontophoresing saline, naloxone or gabazine immediately following termination of EA in twelve animals. In another group of eight animals, the effect of either antagonist was evaluated in the absence of EA to determine the roles of these neurotransmitters in the vagal evoked cellular response. The size of the multibarrel pipette used to record and iontophoretically deliver antagonists limited our ability to characterize preganglionic neuronal activity in these groups of neurons that meet all other criteria including convergence of median, cardiac vagal, and baroreceptor afferent inputs as well as cardiac rhythmicity.

**Statistical Analysis**
Data are presented as means±SEM. Peristimulus histograms were constructed to examine the level of evoked activity, measured as the increase in number of spikes above baseline following stimulation of the cardiac vagal afferents. We stimulated the nerve at a frequency of 2 Hz for a period of time (30 s) and analyzed two consecutive 15 s periods (30 stimuli) of the recording that then were averaged to determine the level of evoked activity. Changes in mean arterial pressure and heart rate are presented as bar histograms. The increase in cellular activity and decreases in blood pressure and heart rate before and after delivery of experimental drugs or saline were compared by one-way repeated measures ANOVA followed post hoc with the Student-Newman Keuls test. In addition, we compared the saline vs. treatment (naloxone and gabazine) groups using a two-way ANOVA. Data were plotted and analyzed with the Kolmogorov-Smirnov test for normal data distribution and normalized when necessary with Sigma plot and Sigma Stat (Jandel Scientific). The 0.05 probability level was used to detect significant differences.

We also evaluated time and frequency relationships between NAmb neuronal activity and arterial blood pressure using pulse-triggered averaging as well as coherence analysis. Time domain analyses involved arterial pulse-triggered averaging. A threshold was set at the systolic phase of the arterial pulse for the former method while the latter used spike height discrimination and waveform recognition to sort action potentials during the evaluation period of 300 s. Averages of the arterial pulse and histograms of NAmb neuronal activity were constructed as in our previous studies.(24; 28)

Frequency domain analysis involved assessment of the coherence between NAmb 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 NAmb discharge and arterial blood pressure were generated with 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(31) 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 NAmb neuronal activity and blood pressure at each frequency. Coherence values of ≥0.5 were chosen to reflect a statistically significant relationship between rVLM spikes and arterial blood pressure.(33)

**Histology**

At the end of each experiment, animals were euthanized under deep α-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 at 60-µm coronal sections. Recording and microinjection sites were reconstructed from the dye spots and microelectrode tracks with the aid of a microscope (Nikon) and software (Corel presentation). The sites were plotted on coronal sections separated by 1.0 and 1.9-mm intervals with respect to the obex.(5)

**Results**

**Effects of EA on PBG evoked reflexes**

The cardiovascular reflex responses to repeated stimulation of cardiopulmonary serotonin receptors with PBG every 10 min were consistent. Furthermore, baseline MAP and heart rate before the onset of each PBG administration were consistent throughout the protocol (Figs 1, left panels). The cardiovascular responses to intravenous PBG were modulated by EA for at least 50 min. Application of EA, which was effective in about 75% of subjects, did not influence baseline blood pressure or heart rate. (25; 28; 33; 36; 39) Saline microinjected into the NAmb did not influence the response to EA (Fig 1, right panels). Gallamine triethiodide, used to inhibit muscle movement during stimulation of P5-6, did not affect the EA response.

**Role of NAmb in cardiopulmonary reflex**
Microinjection of KA that induces prolonged depolarization blockade of neurons in the NAmb reduced the inhibitory PBG-related chronotropic reflex response (Fig 2). Similar to our previous studies employing kainate (25; 28; 38), the action of kainic acid lasted approximately 10 min. However, the PBG-induced blood pressure response was not significantly affected by microinjection of KA into the NAmb.

Roles of Opioid and GABA systems in NAmb PBG-cardiovascular responses during EA

As noted in section on PBG evoked reflexes, stimulation of the median nerves (P5-6) by EA reduced PBG-evoked inhibitory responses for at least 50 min. Microinjection of naloxone transiently increased heart rate by 15±5 beats/min in three cats and decreased heart rate by -13±4 beats/min in three other cats, but overall the heart rate remained constant. Heart rate had returned to normal resting levels by the time PBG was administered. Naloxone reversed the chronotropic action of EA, but did not significantly influence EA’s action on the blood pressure response (Fig 3). The two-way ANOVA confirmed a significant difference in heart rate response to PBG comparing the saline control with naloxone. The duration of naloxone’s action on the EA response lasted for at least 10 min, as shown in a previous study.(33)

Microinjection of gabazine altered baseline heart rate (-6±3 beats/min) transiently in six animal. Heart rate was restored to normal level prior to PBG induced reflex response. Similar to our observations with opioid receptor antagonism, blockade of NAmb GABA_A receptors reversed EA modulation of the bradycardia for at least 10 min but did not significantly alter EA’s action on the PBG-induced depressor response (Fig 4). As with naloxone, two-way ANOVA demonstrated a significant difference in heart rate response to PBG in the saline vs. gabazine group.

Nucleus Ambiguus neuronal activity during EA

Neurons in the parasympathetic nucleus were characterized prior to examination of their responses to repeated stimulation of cardiac vagal nerve and EA. Basal activity was 3.4 ±0.5 spikes/s. We identified 37 NAmb neurons that received convergent input during both P5-6 (median nerves, Fig 5A) and cardiac vagal nerve stimulation. All neurons were responsive to activation of baroreceptors (Fig 5C and F) and displayed cardiac rhythmicity.
(Fig 5B and D). We examined seventeen neurons to determine if they directly projected to the heart. Eight of the seventeen, all of which were responsive to EA could be driven antidromically, displayed high fidelity discharge upon rapid stimulation and a constant latency, and therefore were classified as preganglionic NAmb neurons (Fig 5E). EA reduced the cardiac vagal nerve evoked activity of the preganglionic NAmb neurons for at least 30 min during and after EA (Fig 6B). In the absence of EA, stimulation of the cardiac vagus nerve consistently activated NAmb neurons (Fig 6A).

NAmb neuronal response to PBG and EA

All four of four NAmb neurons were activated by PBG as well as by EA stimulation of the median nerve at P5-6, cardiac vagal afferents and baroreceptors. They also displayed pulse related activity. Figure 7 shows PBG-related activation of a NAmb neuron that also responded to EA.

Role of GABA in NAmb neuronal activity during EA

As noted in section on PBG-cardiovascular responses, we observed that GABA receptors in the NAmb participate in EA modulation of the cardiopulmonary reflex responses. Similarly, GABA_A receptors in eight NAmb neurons were shown to be involved in the neuronal responses to acupuncture. Thus, blockade with gabazine reversed EA inhibition of the neuronal discharge activity activated by stimulation of cardiac vagal afferents (Fig 8 A_2 and Fig 6C). In contrast, gabazine did not influence baseline or evoked activity in the absence of EA (Fig 8 A_1).

Role of opioids in NAmb neuronal activity during EA

Application of EA for 30 min decreased NAmb evoked activity each of four cells. Naloxone consistently reversed the influence of EA while in the absence of EA the antagonist did not alter the increased activity of four other NAmb neurons in response to stimulation of the cardiac vagal branch (Fig 8 B_1 and 2). Naloxone did not alter baseline discharge of these neurons.

Histology
We histologically confirmed that the sites of injection and recording were within the coordinates (3.5 mm lateral and 3.7 mm depth) of the inferior lateral region of the NAmb (Fig 9) as shown in Berman’s atlas(5) by observing microelectrode tracks and location of dye injections. These sites were in the region that is known to contain the majority of cardiac vagal preganglionic neurons in the NAmb.(27; 44)

Discussion

We have shown in a series of studies that EA at acupoints P5-6, which provide significant input to the rostral ventrolateral medulla,(35) inhibits sympthoexcitatory reflex responses and thus lowers elevated blood pressure. The present study extends these observations by demonstrating that EA at the same acupoints also is capable of modulating inhibitory cardiovascular responses, especially the negative chronotropic response, processed in the NAmb. In this regard, activation of cardiopulmonary 5-HT3 receptors with PBG-induced decreases blood pressure and heart rate through a parasympathoexcitatory reflex was reduced by 30 min of low frequency, low intensity EA at P5-6. This form of EA is known to activate both Group III and IV fibers in the median nerve.(22; 37) Both opioids and GABA in the NAmb contributed to EA-related modulation of neural activity as well as the reflex cardiopulmonary depressor response. This study is the first demonstration that acupuncture is capable of modulating reflexes that involve parasympathetically mediated chronotropic inhibitory responses through its action in the NAmb.

Cardiovascular neurons in the ventrolateral NAmb region were activated by stimulation at P5-6 and PBG. This study is the first to examine functional properties of NAmb neurons during activation of cardiac vagal afferents by the Bezold-Jarish reflex. Many of the neurons influenced by EA were preganglionic cardiac vagal neurons. The onset and duration of the effects of EA on the cardiac parasympathetic-evoked NAmb neuronal activity in the two groups studied during receptor blockade were similar in magnitude to the influence of acupuncture on reflex decreases in blood pressure and heart rate. Thus, it appears that EA acts on cardiovascular parasympathetic preganglionic and possibly interneurons that
participate in NAmb processing of cardiopulmonary inhibitory reflexes during somatic input evoked by EA.

We observed no changes in basal heart rate and NAmb neuronal activity following opioid receptor blockade. Furthermore, blockade of opioid receptors did not alter cardiac vagal responses to vagal afferent stimulation, in the absence of EA. On the other hand, opioids released during EA inhibited cardiopulmonary-induced activation of parasympathetic outflow. We recently have shown that preganglionic cholinergic NAmb neurons activated with acupuncture are located in close proximity to enkephalinergic fibers.(13) Although in this anatomical study we were unable to determine if the EA-activated cholinergic (preganglionic) neurons were of cardiac origin(13), enkephalin is known to be present in fibers in close proximity to cardiac vagal neurons in the NAmb.(4; 48) Thus, during EA opioids, particularly enkephalins, in the NAmb appear to regulate cardiac chronotropic activity by modulating (inhibiting) vagal activity.

We demonstrated that GABA in the NAmb was involved in processing somatic input during EA but did not alter basal heart rate and neuronal activity. Application of a GABA antagonist into the NAmb did not alter cardiac vagal evoked activity while the bradycardia induced by activation of cardiopulmonary afferents was inhibited by EA through a GABAergic mechanism. Microinjection of GABA into the NAmb induces bradycardia suggesting that GABA tonically inhibits cardiac parasympathetic outflow from the NAmb.(26; 29; 46) Thus, in addition to opioids, a GABA mechanism during EA contributes to modulation of neurons in the NAmb that, in turn, reduce vagal outflow and the negative chronotropic response to PBG-induced stimulation of vagal afferents.

Neurons in the NAmb that were evaluated in this study and shown to participate in the cardiopulmonary Bezold-Jarisch reflex were classified as cardiovascular since they responded to baroreceptor input, had a pulse related rhythm and showed strong coherence to blood pressure. Baroreceptor-induced modulation of heart rate is known to be processed in the NAmb.(26) In addition to input from the median and cardiac vagal nerves, PBG stimulated some and many were preganglionic cardiac vagal neurons, since they could be driven antidromically from the cardiac vagus nerve. Due to technical limitations we were unable to attempt antidromic stimulation in a number of NAmb neurons, which were
evaluated for neurotransmitter mechanisms of EA’s action. We therefore cannot exclude the possibility that some neurons functioned as inter- rather than preganglionic neurons.

The NAmb is located in close proximity to the cVLM and it is thus important to be sure that we targeted the former rather than the latter nucleus. We used both physiological as well as anatomical data to confirm that the injections were confined to the NAmb. With respect to our physiological proof, we recently have shown that blockade of neurons in the cVLM during a depressor reflex in rats reduces the decrease in blood pressure but does not influence heart rate. On the other hand, receptor blockade in the NAmb reduces heart rate but not blood pressure. Similarly, in the current study, blockade with naloxone or gabazine reversed the heart rate but not the blood pressure aspect of the reflex, suggesting that we influenced the NAmb and not the cVLM. Moreover, the microinjection or recording electrodes were advanced ventrally to a position of 3.7 to 3.9 mm from the dorsal surface of the medulla, coordinates that access the NAmb and not the cVLM according to Berman’s cat atlas. These results support proper positioning of the electrode in the NAmb, and most specifically in the ventrolateral region of the NAmb, which has the greatest concentration of vagal preganglionic neurons, rather than in the cVLM.

EA-related restoration of blood pressure responses to cardiopulmonary reflex stimulation was not significantly influenced by blockade in the NAmb, suggesting that distinct central mechanisms and regions unique to EA’s action, such as its influence on sympathetic vasomotor centers like the rVLM and raphé nuclei in the brain stem may be involved in this aspect of EA modulation. Similar to the NAmb, GABA and opioids in the rVLM and raphé nuclei contribute to the inhibitory effects of EA on sympathoexcitatory reflex responses. The cVLM also regulates vasomotor tone through a GABAergic mechanism, although its importance in EA has yet to be demonstrated. Thus, a number of cardiovascular centers regulating vasomotor tone process acupuncture input that modulates sympathoexcitatory autonomic responses. Preliminary data from our group also has shown that acupuncture likely also influences these sympathetic cardiovascular regions during sympathoinhibitory activity. Machado and Brody have suggested that a neuronal connection exists between the NAmb and sympathoexcitatory centers. Furthermore, the nucleus tractus solitarii, the initial synapse in cardiopulmonary reflex inhibition projects to the cVLM and rVLM. Hence, it is likely that EA regulates blood
pressure (as opposed to heart rate) mainly through its action on sympathetic rather than parasympathetic centers in the brain stem.

Perspective
Acupuncture, which is effective in about 70-80% of subjects, is known to influence cardiovascular function and eventually may serve a role in medical conditions associated with low blood pressure. For example, EA reverses hemorrhage-induced hypotension, possibly by increasing venous return through enhanced vasomotor tone and the muscle pump.(32) Similarly, in a more clinically related study, acupuncture has been shown to elevate blood pressure in patients with shock.(6) The underlying central neural mechanisms of the action of EA in hypotension induced by hemorrhage or shock have not previously be investigated. The current study utilized a model of hypotension involving strong activation of cardiopulmonary afferents and the parasympathetic nervous system to lower heart rate shares many features of vasovagal syncope. We demonstrated that convergence of somatic and parasympathetic afferent inputs on common preganglionic neurons (and possibly interneurons) in the NAmb through both opioid and GABAergic processing mechanisms can participate in restoration of blood pressure. These, may serve as mechanisms underlying acupuncture’s beneficial action in hypotension, vasovagal syncope and shock. Vasovagal syncope is the most common cause of transient unconsciousness.(45) It is mediated largely by activation of a cardiopulmonary mediated reflex(2) and hence in susceptible individuals who repeatedly experience this problem and are at risk for syncope; EA might serve as a therapeutic option.

Opioid and GABA blockade reversed the bradycardia but not the depressor portion of EA-PBG reflex modulation. This brings into question the role of the NAmb in the hypotensive portion of the response. Since cardiac output is a function of heart rate and stroke volume, EA related modulation of heart rate has the potential to influence cardiac output and hence blood pressure. Furthermore, the parasympathetic system is essential to many hypotensive responses, for example those occurring during hemorrhage and vasovagal syncope.(17) EA thus has the potential to modify these untoward reflexes associated with stimulation of cardiopulmonary afferents through its actions in the NAmb.
Conclusion

Electroacupuncture for 30 min reduces the augmented activity of the parasympathetic-related preganglionic neurons in the NAmb to restore heart rate during PBG stimulation of the cardiopulmonary afferents. Opioids and GABA are two neuromodulatory systems that underlie EA’s action in this region.

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Legends

Figure 1. Decreases in blood pressure and heart rate were reduced by electroacupuncture (EA). Left panels are control data showing consistent blood pressure and heart rate responses to repeated cardiopulmonary 5-HT₃ receptor stimulation with phenylbiguanide (PBG) iv every 10 min. Panels on the right display the effects of EA on these vagal excitatory cardiovascular reflex responses. Baseline blood pressure and heart rate are shown above each bar as means±SEM.

Figure 2. Bar histograms display decreased blood pressure and heart rate responses to PBG before and after microinjection of kainic acid in nucleus ambiguus (NAmb). KA transiently reduced the reflex chronotropic response (*, P<0.05). Panels a-c and d-f show the parasympathoexcitatory hemodynamic and negative chronotropic reflex responses before and after KA in an individual animal. Letters a-f shown in the bars correspond with the original tracing of blood pressure and heart rate. Baseline blood pressure and heart rate are shown above each bar as means±SEM.

Figure 3. Role of opioid in NAmb in the action of EA on bradycardia. Bar histograms represent decreases in blood pressure and heart rate (*, P<0.05) following repeated administration of PBG, compared to control pre-EA. Naloxone in NAmb reversed EA inhibition of bradycardia following cardiopulmonary reflex (†, P<0.05, comparing panels f and g). Letters a-h shown in the bars correspond to the original tracing of blood pressure and heart rate. Baseline blood pressure and heart rate are shown above each bar as means±SEM.

Figure 4. GABA contributed to action of EA on decrease heart rate. The PBG reflexes were reduced during action of EA on blood pressure and heart rate responses (*, P<0.05), compared to control pre-EA. Gabazine microinjected into NAmb reversed the EA effect on bradycardia (†, P<0.05) comparing panels f and g but not the decreased blood pressure responses. Letters a-h shown in the bars correspond to the original tracings of blood pressure and heart rate. Baseline blood pressure and heart rate are shown above each bar in means±SEM.
Figure 5. Example of a premotor parasympathoexcitatory cardiac neuron in the nucleus ambiguus (NAmb) receiving median nerve (MN) and cardiac vagal convergent input that also displayed barosensitivity and rhythmicity with the cardiac cycle. Panel A shows a peristimulus histogram and representative neurogram of median nerve evoked activation following stimulation artifacts. The neuron displayed cardiac rhythmicity with pulse triggered averaging (Panel D) and a coherence of 0.81 at a frequency of 4.2 Hz (Panel B). Barosensitivity, determined with phenylephrine (PE) to elevate blood pressure, increased the discharge frequency (Panel C). Group data are shown in the histograms (Panel F). The neuron in the NAmb projecting to the heart was characterized by collision testing through antidromic stimulation of the cardiac vagal branch (Panel E). Neuron was antidromically activated with 2 Hz, 4V and 0.5 s. Critical interval was 32 ms. Symbols ● and * indicate the stimulation artifacts of the MN and vagal cardiac nerve, respectively. Middle panel, antidromic spike is absent during collision with orthodromically MN evoked spike. An action potential is displayed below the neurograms.

Figure 6. Bar histograms display neuronal activity in the nucleus ambiguus (NAmb) during repeated stimulation of the cardiac vagal afferents every 10 min. Panel A shows consistent evoked responses. Panel B shows reduced activity during and after stimulation of acupoints P5-P6 for 30 min. Peristimulus histograms a-c corresponding with the letters in the bars show cardiac vagal evoked activity of a neuron before, during and after EA. Panel C shows that saline did not influence the action of EA on NAmb activity. *, indicates EA decreased evoked discharge rate, P<0.05.

Figure 7. PBG sensitive cardiovascular neurons in the NAmb activated with cardiac vagal afferents were responsive to EA. Panel A shows increased discharge rate associated with the PBG–induced vasodepressor response. Panel B displays EA-inhibition of repeated cardiac vagal nerve evoked activity of NAmb neurons sensitive to intravenous administration of PBG.
Figure 8. Evoked activity in the nucleus ambiguus (NAmb) reduced during EA involves GABA_A and opioid receptors. Neither gabazine nor naloxone influenced the PBG induced evoked responses (Panels A1 and B1). On the other hand, blockade of both GABA_A and opioid receptors during the inhibitory action of EA abolished acupuncture’s action on neuronal activity. Letters a-c shown in the bars correspond to the peristimulus histograms.

Figure 9. Composite map displays the sites of microinjection, iontophoresis and extracellular recordings in the NAmb. *, kainic acid; ■, naloxone; ▲, gabazine; ●, cardiac vagal nerve evoked activity; △, saline; □, outside. Photomicrograph displays the blue dyed track of micropipette insertion. Most ventral aspect of the blue dye represents the site of microinjection shown with an arrow.
Reference


EA

MAP (mmHg)

HR (beats/min)

Saline (NAmb)

n=5

n=6

130±13 132±9 135±8 138±11 131±8 139±8 132±8 134±7 121±11 125±11

174±25 175±22 176±24 182±29 172±12 179±19 177±20 188±21 163±14 167±12

122±6 111±4 115±7 123±8 119±6 116±6 115±5 109±3 112±8 116±6

183±19 210±17 219±14 219±15 213±11 215±11 212±12 214±15 214±12 214±15
A.

BP (mmHg)

HR (beats/min)

spikes/s

0 60 120 s

PBG iv

7 ms

B.

n=4

NAmb spikes/30 stim

EA

* * *
A1. Gabazine (NAmb) n=4

B1. Naloxone (NAmb) n=4

A2. Gabazine (NAmb) n=4

B2. Naloxone (NAmb) n=4

Cardiac vagal nerve
7 spikes/30 stim

Cardiac vagal nerve
2 spikes/30 stim

Cardiac vagal nerve
5 spikes/30 stim