Gamma aminobutyric acid and glycine receptors in the nucleus ambiguus mediate tachycardia elicited by chemical stimulation of the hypothalamic arcuate nucleus

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ABSTRACT

We have previously reported that stimulation of the hypothalamic arcuate nucleus (ARCN) by microinjections of N-Methyl-D-aspartic acid (NMDA) elicits tachycardia which is partially mediated via inhibition of vagal inputs to the heart. The neuronal pools and neurotransmitters in them mediating tachycardia elicited from the ARCN have not been identified. We tested the hypothesis that the tachycardia elicited from the ARCN may be mediated by inhibitory neurotransmitters in the nucleus ambiguus (nAmb). Experiments were done in urethane-anesthetized, artificially ventilated, male Wistar rats. In separate groups of rats, unilateral and bilateral microinjections of muscimol (1 mM), gabazine (0.01 mM) and strychnine (0.5 mM) into the nAmb significantly attenuated tachycardia elicited by unilateral microinjections of NMDA (10 mM) into the ARCN. Histological examination of the brains showed that the microinjections sites were within the targeted nuclei. Retrograde anatomical tracing from the nAmb revealed direct bilateral projections from the ARCN and hypothalamic paraventricular nucleus to the nAmb. The results of this study suggest that tachycardia elicited by stimulation of the ARCN by microinjections of NMDA is mediated via GABA$_A$ and glycine receptors located in the nAmb.
Key words

Gabazine, muscimol, N-methyl-D-aspartic acid, strychnine, tachycardia
INTRODUCTION

The hypothalamic arcuate nucleus (ARCN), located bilaterally at the base of the caudal 2/3 of the third ventricle, plays an important role in central cardiovascular regulation in normal and pathological states (41). We have recently shown that microinjections of N-methyl-D-aspartic acid (NMDA) into the ARCN elicited an increase in heart rate (HR) in rats regardless of whether the baroreceptors were intact or denervated (26-27, 36). The tachycardic responses elicited from the ARCN are mediated via the release of glutamate and α-MSH in the hypothalamic paraventricular nucleus (PVN) because blockade of ionotropic glutamate receptors (iGLURs) and melanocortin 3/4 receptors (MC3/4Rs) in the ipsilateral PVN attenuated these responses (27). In this context, it may be noted that proopiomelanocortin (POMC) immunoreactive cells are present in the ARCN (27). POMC is the precursor of α-MSH and a subpopulation of POMC neurons in the ARCN is glutamatergic (29, 33).

The increases in HR elicited by NMDA-induced stimulation of the ARCN are partially mediated via activation of sympathetic input to the heart (36). The activation of sympathetic input to the heart, following the stimulation of the ARCN, is mediated via projections of the PVN to the intermediolateral cell column of the spinal cord (IML) or projections of the PVN to the rostral ventrolateral medullary pressor area (RVLM) which, in turn, projects to the IML (14, 19, 36, 39, 40). Glutamate is implicated as the neurotransmitter in the IML because blockade of iGLURs in the IML attenuated the tachycardic responses elicited from the ARCN (36).

The tachycardic responses elicited NMDA-induced stimulation of the ARCN is also partially mediated via inhibition of the vagal input to the heart (36). The neuronal pools mediating the inhibition of vagal input to the heart in response to ARCN stimulation and the neurotransmitters and receptors involved in these neuronal pools have not been identified.
Although cardiac vagal neurons (CVNs) in the rat are located in the dorsal motor nucleus of the vagus as well as the nucleus ambiguus (nAmb), the predominant vagal input to the heart is provided by the CVNs located in the nAmb. The axons of the CVNs located in the nAmb pass through the vagus nerves and synapse on cardiac ganglia located in the atrioventricular node and fat tissue surrounding the sinoatrial node. Typically CVNs located in the nAmb are silent and need excitatory and inhibitory synaptic inputs for their activation and inhibition, respectively. The CVNs in the nAmb are known to receive gamma-aminobutyric acid (GABA) and glycine inhibitory inputs. Based on this information, we hypothesized that GABA and glycine may be released in the nAmb in response to ARCN stimulation causing an inhibition of CVNs via GABA and glycine receptors, respectively. Consequently, the parasympathetic input to the heart is decreased resulting in tachycardia. This hypothesis was tested in this paper.
METHODS

General procedures

Adult male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 300–360 g were used. A total number of 69 rats were used. The distribution of rats in different groups was as follows: microinjections of NMDA into the ARCN (n = 9), inhibition of nAmb neurons using muscimol (n = 15), blockade of GABA_A receptors in the nAmb using gabazine (n = 17), blockade of glycine receptors using strychnine in the nAmb (n = 10), combined blockade of GABA_A and glycine receptors in the nAmb (n = 5), blockade of iGLURS in the nAmb (n = 7) and retrograde tracing studies (n = 6).

The animals were housed under controlled conditions with a 12h:12h light-dark cycle. Food and water were available to the animals ad libitum. The experimental protocols, designed according to the NIH “Guide for the Care and Use of Laboratory Animals”, were reviewed and approved by the Institutional Animal Care and Use Committee of this university.

Details of all the procedures used in the current study are reported in our earlier publications (1, 6, 8-10, 24-27, 36). The rats were initially anaesthetized with inhalation of isoflurane (2-3% in 100% oxygen), using a vaporizer (Fluotec-3, Cyprane Ltd., UK), in order to cannulate the trachea and the femoral vein and artery on one side. Isoflurane administration was then continued via the trachea using a rodent ventilator (model 683, Harvard Apparatus, Holliston, MA, USA). A solution of urethane (800 mg/ml) was then injected intravenously in 6-9 aliquots (each 0.05-0.1 ml containing 40-80 mg of urethane) at 2 min intervals. The inhalation of isoflurane was discontinued after the administration of 4-5 aliquots of urethane. The injection of urethane was completed within 12-18 min. The total dose of urethane injected was 1.2-1.4 gm/kg, i.v. Adequate depth of anesthesia, indicated by the absence of an increase in blood
pressure (BP) and/or withdrawal of the limb in response to pinching of a hind paw, was tested periodically throughout the experiment. The end-tidal CO$_2$ was measured continuously from expired gases, using an infrared CO$_2$ analyzer modified for use in small animals (Micro-Capnometer, Columbus Instruments, Columbus, OH, USA). The frequency and tidal volume were adjusted on the ventilator to maintain the end-tidal CO$_2$ at 3.5-4.5%. The rectal temperature was monitored using a rectal probe (RET-1) connected to temperature controller (model TCAT-2A, Physitemp Instruments, Clifton, NJ, USA) and was maintained at 37 ± 0.5°C. BP and HR were recorded on a computer hard drive using 1401 plus A/D converter and Spike 2 software (Cambridge Electronic Design Ltd., Cambridge, UK).

Microinjections into the ARCN

The rats were placed in a prone position in a stereotaxic instrument (David Kopf Instruments, Tajunga, CA, USA) with bite bar 18 mm below the interaural line. A hole (8-10 mm in diameter) was drilled in the midline at the junction of the two parietal bones caudal to the bregma. Our experiments required microinjections into the ARCN and the nAmb in the same animal. Therefore, a double barrel glass-micropipette (tip size 20–40 μm), used for microinjections into the ARCN, was mounted on a micromanipulator (David Kopf Instruments) and fixed at an acute angle (22°) pointing caudally. The barrels in the micropipette were connected to a picospritzer (General Valve, Fairfield, NJ, USA). The glass-micropipettes were inserted into the brain tissue through the previously made hole in the parietal bones on either side of the midline. In order to approach the ARCN, the following coordinates were used: 1.72 – 4.36 mm caudal to the bregma, 0.2-1 mm lateral to the midline and 8.8-10.1 mm ventral to the dura. Using this approach the tip of the micropipette reached the ARCN at the following coordinates: 1.92-3.72 caudal to the
bregma, 0.2-0.6 lateral to the midline and 9.6-10.1 ventral to the dura. Using a modified binocular horizontal microscope (model PZMH, WPI, FL, USA) with a graduated reticule in one eye-piece, the volume of microinjection was visually confirmed by the displacement of fluid meniscus in the micropipette-barrel. Microinjections of NMDA (10 mM) were used to identify the ARCN (1, 11, 26-28, 36). In this and other series of experiments, unless indicated otherwise, the duration of microinjections was 5-10 sec and microinjections of artificial cerebrospinal fluid (aCSF, pH 7.4) were used as controls. As mentioned earlier in this section, our experiments required microinjections into the ARCN and the nAmb in the same animal. Therefore, the interval between microinjections into the ARCN and nAmb was at least 20 min in order to allow the HR response elicited from the ARCN to return to basal level before the pharmacological manipulation in the nAmb were attempted.

Microinjections into the nAmb

Microinjections into the nAmb were made using a dorsal approach (6, 8-10). The dorsal neck muscles and part of the occipital bone were removed and the medulla was exposed by incisions in the atlanto-occipital membrane and dura. Four-barrel micropipettes, tip size 20-40 µm, were lowered into the nAmb perpendicularly. The coordinates for the nAmb were: 0.12 caudal to 0.64 mm rostral with reference to the calamus scriptorius (CS), 1.8-2 mm lateral to the midline and 2-2.4 mm deep from the dorsal surface of the medulla. The remaining procedure for microinjections into the nAmb was similar to that described for microinjections into the ARCN except that L-glutamate (L-Glu, 5 mM), instead of NMDA, was used to identify the nAmb sites eliciting bradycardia.
Retrograde tracing of ARCN projections

The work station and the stereotaxic assembly were sanitized by Clorox spray. The rats were anesthetized with intraperitoneal (i.p.) injections of pentobarbital sodium (50 mg/kg) and fixed in a prone position in the stereotaxic instrument. All the surgical instruments were sterilized using an autoclave. The surgical procedure for approaching the nAmb was identical to that described under “Microinjections into the nAmb”. A microinjection of either green retrobeads solution (20 nl, 10% w/v) (n = 4) or fluorogold (2%, 5 nl) (n = 2) was made into the nAmb. After completing the microinjection, the exposed brain surface was covered with a small piece of absorbable gelatin sponge (Surgifoam, Ethicon Inc., Somerville, NJ, USA) and the skin over the wound was sutured. After the recovery from pentobarbital anesthesia, the rats survived for 7-12 days. The rats were administered an antibiotic (cefazolin, 30 mg/kg) subcutaneously twice a day for 3 days and one dose of a slow release dosage form of an analgesic (buprenorphine SR, 1 mg/kg). On the day of sacrifice, the animals were deeply anesthetized with pentobarbital and perfused with heparinized normal saline followed by 2% paraformaldehyde solution. The brains were removed and stored for 48 hrs in 2% paraformaldehyde. After the completion of the fixation procedure, left or right side of the brain surface was marked by a superficial cut and serial sections of the brain containing the hypothalamus and nAmb were cut (40 µm) in a vibratome (1000 Plus Sectioning System, The Vibratome Company, St. Louis, MO, USA). The sections were mounted on subbed slides, covered with Citifluor mountant medium (Ted Pella Inc., Redding, CA, USA) and coverslipped. The microinjection sites of green retrobeads solution (Amax = 460 nm, Emax = 505 nm) and of fluorogold (Amax = 360 nm, Emax = 515 nm) in the nAmb and the retrogradely-labeled cells in the brain sites of our interest were visualized under a microscope (model AX70, Olympus Provis, Middlebush, NJ, USA). In retrograde tracing studies, the brain...
sites of our interest included the ARCn, caudal ventrolateral medullary depressor area (CVLM), RVLM, nucleus tractus solitarius (NTS) and PVN. The brain sections were compared with a standard atlas (38) and photographed using Neurolucida software (version 7.5, MicroBrightField Inc., Williston, VT, USA).

Histological verification of microinjection sites

At the end of the experiment, microinjections of green retrobeads (0.2% w/v) were made into the nAmb (20 nl) and ARCn (30 nl) in order to mark the microinjection sites. The animals were then perfused with heparinized normal saline and paraformaldehyde, the brains were removed and prepared for making sections as described under “Retrograde tracing of ARCn projections”. The microinjection sites of green retrobeads in the nAmb and ARCn were visualized under a microscope, photographed using Neurolucida software and compared with a standard atlas (38).

Drugs and chemicals

The following drugs and chemicals were used in this study: D-AP7 (NMDA receptor antagonist) (D(-)-2-amino-7-phosphono-heptanoic acid), gabazine bromide (GABA_A receptor antagonist), L-glutamate monosodium (L-Glu), isoflurane, NBQX disodium salt (non-NMDA receptor antagonist) (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-[f]quinoxaline-7-sulphonamide disodium), muscimol hydrobromide, strychnine hydrochloride and urethane. Green retrobeads solution and fluorogold were procured from Lumafluor Corp. (Durham, NC, USA) and Fluorochrome Inc., (Denver, CO, USA), respectively. Isoflurane was purchased from Piramal Critical Care (Bethlehem, PA, USA). All other drugs were purchased from Sigma-Aldrich Chemicals (St.
Louis, MO, USA). All of the solutions for the microinjections were freshly prepared in aCSF (pH 7.4).

**Statistical analyses**

Maximum changes in mean arterial pressure (MAP) and HR in response to microinjections of different drugs were expressed as the means and standard error of the means (S.E.M.). One-way analysis of variance (ANOVA) followed by Tukey-Kramer’s multiple comparison was used to evaluate the significance of differences in maximum changes in MAP and HR elicited by microinjections of different drugs in different groups of rats. Student's paired t-test was used for comparison of the following responses: decreases in MAP and increases in HR induced by the microinjections of NMDA into the ARCN and decreases in HR elicited by microinjections of D-AP7, gabazine, L-Glu, NBQX and strychnine and increases in HR by microinjections of muscimol into the nAmb. The differences were considered significant at p < 0.05 in all cases.
RESULTS

Baseline values for MAP and HR in urethane-anesthetized rats were 101.9 ± 3.4 mmHg and 399.5 ± 4.5 bpm, respectively (n = 69).

Effect of NMDA microinjection into the ARCN

In this and other series of experiments, the ARCN was always identified by microinjections of NMDA (10 mM), which elicited decreases in MAP (19 ± 1 mmHg) and increases in HR (46 ± 3 bpm) (n = 9) in non-barodenervated rats (26). The time intervals between the microinjection and beginning of the response (onset) and maximum response (peak) and the end of response (duration) were noted. The onset, peak and duration of the depressor responses to microinjections of NMDA (1 mM) were 14.1 ± 2.4 sec, 2.8 ± 0.5 min and 10.1 ± 1.6 min, respectively. The onset, peak and duration of the tachycardic responses (16 ± 3 sec, 3 ± 1 min and 13 ± 2 min, respectively) were not statistically different from those of depressor responses. These time intervals are similar to those previously reported by us and others following the microinjections of NMDA into the ARCN or PVN (24, 26, 30). In the ARCN, all microinjections were unilateral and the volume of microinjections was 30 nl. Microinjections of aCSF (30 nl) into ARCN elicited no cardiovascular responses.

Effect of muscimol microinjections into nAmb on NMDA-induced HR responses from ARCN

In one group of rats (n = 9), the ARCN was identified by microinjections (30 nl) of NMDA (10 mM); as mentioned in the preceding paragraph, increases in HR were elicited. Twenty min later, when the effects of NMDA in the ARCN had abated, ipsilateral nAmb was identified by microinjections of L-Glu (5 mM, 20 nl) in the same rats; decreases in HR (76.4 ± 9.3 bpm) were
elicited within 2 ± 0.1 sec and this effect lasted for 74.8 ± 10.7 sec. In this and other experiments, microinjections of aCSF (20 nl) into nAmb elicited no cardiovascular responses. Next, muscimol (1 mM, 20 nl) was microinjected into the nAmb. Muscimol was used as a pharmacological tool to inhibit the nAmb neurons in this experiment. The concentration of muscimol used in this study was selected from one of our previous reports (9-10). Unilateral microinjections of muscimol into the nAmb elicited an increase in HR (31.4 ± 4.1 bpm); the onset and duration of this effect were 4.9 ± 0.9 sec and 6.1 ± 1 min, respectively. At this time, microinjection of NMDA into the ARCN was repeated. The increases in HR induced by NMDA in the ARCN were significantly (p < 0.0001) reduced; the increases before and after the unilateral microinjections of muscimol into the ipsilateral nAmb were 46.1 ± 3.2 and 25.1 ± 2.2 bpm, respectively (Fig. 1A-B).

In another group of rats (n = 6), the ARCN was identified by microinjections of NMDA as mentioned earlier. Twenty min later, nAmb was identified bilaterally by microinjections of L-Glu (5 mM, 20 nl each side) in the same rats. Next, muscimol was microinjected into the nAmb bilaterally (1 mM, 20 nl each side). Bilateral microinjections of muscimol into the nAmb elicited an increase in HR (46.6 ± 1.8 bpm) within 5.9 ± 0.4 sec and this effect lasted for 12.4 ± 0.9 min. At this time, the increases in HR induced by NMDA in the ARCN were significantly reduced; the increases before and after the bilateral microinjections of muscimol into the nAmb were 55.8 ± 3.4 and 10.1 ± 1.3 bpm (Fig. 1C-D), respectively (p < 0.0001). The attenuation of tachycardic responses elicited by microinjections of NMDA into the ARCN by bilateral microinjections of muscimol into the nAmb was significantly greater (p < 0.001) when compared to the attenuation caused by unilateral microinjection of muscimol into nAmb. Typical tracings of the effect of
bilateral microinjections of muscimol into the nAmb on the tachycardic responses elicited from the ARCN are shown in Figs. 1E-J.

**Effect of microinjections of gabazine into nAmb on NMDA-induced HR responses from ARCN**

In one group of rats (n = 9), the ARCN was identified by microinjections of NMDA. Twenty min later, ipsilateral nAmb was identified by microinjections of L-Glu (5 mM, 20 nl) in the same rats. Next, gabazine (0.01 mM, 20 nl) was microinjected into the ipsilateral nAmb. The concentration of gabazine used in this study was selected from one of our previous reports (28). Unilateral microinjections of gabazine into the nAmb elicited a decrease in HR (17.7 ± 1.1 bpm); the onset and duration of this effect were 6.6 ± 0.8 sec and 5.7 ± 0.7 min, respectively. The increases in HR induced by NMDA in ARCN were significantly (p < 0.001) attenuated by unilateral microinjections of gabazine into the nAmb; increases in HR elicited by ARCN stimulation before and after the microinjections of gabazine into the ipsilateral nAmb were 56.7 ± 9.6 and 33 ± 5.4 bpm, respectively (Fig. 2A-B).

In another group of rats (n = 8), the ARCN was identified by microinjections of NMDA. Twenty min later, nAmb was identified bilaterally by microinjections of L-Glu (5 mM, 20 nl) in the same rats. Next, gabazine was microinjected into the nAmb bilaterally (0.01 mM, 20 nl each side). Bilateral microinjections of gabazine into the nAmb elicited a decrease in HR (21.7 ± 1.8 bpm) within 7.4 ± 0.5 sec and this effect lasted for 10.6 ± 1.6 min. At this time, the increases in HR induced by NMDA in the ARCN were significantly reduced; the increases in HR before and after the bilateral microinjections of gabazine into the nAmb were 50.5 ± 4.3 and 10.6 ± 1 bpm (p < 0.0001), respectively (Fig. 2C-D). The attenuation of tachycardic responses elicited from the ARCN by NMDA was significantly (p < 0.01) greater with bilateral microinjections of gabazine.
into the nAmb when compared to unilateral microinjection of gabazine into nAmb. Typical
tracings of the effect of bilateral microinjections of gabazine into the nAmb on the tachycardic
responses elicited from the ARCN are shown in Figs 2E-2J.

Effect of strychnine microinjections into nAmb on NMDA-induced HR responses from ARCN

In one group of rats (n = 5), the ARCN was identified by microinjections of NMDA. Twenty min
later, ipsilateral nAmb was identified by microinjections of L-Glu (5 mM, 20 nl) in the same rats.
Next, strychnine (0.5 mM, 20 nl) was microinjected into the ipsilateral nAmb. The concentration
of strychnine used in this study was selected from one of our previous reports (7). Unilateral
microinjections of strychnine into the nAmb elicited a decrease in HR (14.3 ± 1.8 bpm); the
onset and duration of this effect were 5.9 ± 0.8 sec and 5.8 ± 0.7 min, respectively. At this time,
increases in HR elicited by microinjections of NMDA into the ARCN were significantly (p <
0.05) reduced; the increases in HR before and after the microinjections of strychnine into the
ipsilateral nAmb were 43.8 ± 7 and 23.4 ± 2.8 bpm, respectively (Fig. 3A-B).

In another group of rats (n = 5), the ARCN was identified by microinjections of NMDA. Twenty min later, the nAmb was identified bilaterally by microinjections of L-Glu (5 mM, 20 nl each side) in the same rats. Next, strychnine (0.5 mM, 20 nl each side) was microinjected into the nAmb bilaterally. Bilateral microinjections of strychnine into the nAmb elicited a decrease in HR (22.9 ± 2.3 bpm) within 7.1 ± 0.6 sec and the duration of this effect was 12.3 ± 1.2 min. At this time, the increases in HR induced by NMDA in the ARCN were significantly (p < 0.01) reduced; the increases before and after the bilateral microinjections of strychnine into the nAmb were 49.2 ± 6.3 and 12.2 ± 0.5 bpm, respectively (Fig. 3C-D). The attenuation of NMDA-induced tachycardia in the ARCN elicited by bilateral microinjections of strychnine into the
nAmb was significantly (p < 0.05) greater when compared to the attenuation caused by unilateral microinjections of strychnine into nAmb. Typical tracings of the effect of bilateral microinjections of strychnine into the nAmb on the tachycardic responses elicited from the ARCN are shown in Figs. 3E-3J.

Effect of combined blockade of GABA$_A$ and glycine receptors in the nAmb on NMDA-induced HR responses from ARCN

In one group of rats (n = 5), the ARCN was identified by microinjections of NMDA. Twenty min later, ipsilateral nAmb was identified by microinjections of L-Glu (5 mM, 20 nl) in the same rats. Next, gabazine (0.01 mM) and strychnine (0.5 mM) (20 nl each) were microinjected sequentially within 2-3 min into the ipsilateral nAmb. Combined microinjections of gabazine and strychnine into the nAmb elicited a decrease in HR (20.2 ± 2.2 bpm); the onset and duration of this effect were 5.8 ± 0.4 sec and 6.9 ± 0.7 min, respectively. At this time, increases in HR elicited by microinjections of NMDA into the ARCN were significantly (p < 0.001) reduced; the increases in HR before and after the combined microinjections of gabazine and strychnine into the ipsilateral nAmb were 43.2 ± 4.8 and 16.8 ± 6.1 bpm, respectively (Fig. 4A-B).

Effect of blockade of iGLURS in the nAmb on NMDA-induced HR responses from ARCN

In a group of rats (n = 7), the ARCN was identified by microinjections of NMDA. Twenty min later, ipsilateral nAmb was identified by microinjections of L-Glu (5 mM, 20 nl) in the same rats. Next, D-AP7 (5 mM) and NBQX (2 mM) were microinjected (20 nl each) into the ipsilateral nAmb. The concentrations of D-AP7 and NBQX used in this study were selected from one of our previous publications (9). Unilateral microinjections of the iGLUR antagonists into the
nAmb elicited a decrease in HR (16.4 ± 2.1 bpm); the onset and duration of this effect were 2.8 ± 1 sec and 5.9 ± 0.5 min, respectively. Blockade of iGLURs resulted in significantly (p < 0.05) reduced HR increases to NMDA microinjections into the ARCN; the increases in HR before and after the blockade of iGLURs in the ipsilateral nAmb were 54.6 ± 6.2 and 37.9 ± 3.7 bpm, respectively (Fig. 4C-D).

Histological verification of microinjection sites in the ARCN

A typical microinjection site in the ARCN marked by green retrobeads is shown in Fig. 5A. The spot was in the ARCN at a level 2.28 caudal to the bregma and the center of the spot was 0.35 mm lateral to the midline and 9.7 mm deep from the dura. The microinjection sites in the ARCN at different rostro-caudal levels (3.72-1.92 caudal to the bregma) are shown in Figs. 5B-5G in which each dark spot represents a site in one animal (all microinjection sites are not shown because of overlapping). The rats, in which the diffusion sphere of the marker was not within the boundaries of the ARCN, as shown in a standard atlas (38), were not included in the study.

Histological verification of microinjection sites in the nAmb

A typical microinjection site in the nAmb marked with green retrobeads is shown in Fig. 6A; the center of the spot in this figure was located 0.24 mm rostral to the CS, 1.9 mm lateral to the midline and 2.1 mm deep from the dura. Diagrams showing other microinjection sites in the nAmb, at different rostro-caudal levels (0.64 mm rostral to 0.12 mm caudal to the CS) are shown in Figs. 6B-6H. In these diagrams, each dark spot represents a microinjection site in one animal (all microinjection sites are not shown because of overlapping). The rats in which the green
retrobeads or fluorogold microinjections were not within the boundaries of the nAmb, as shown in a standard atlas (38), were not included in the study.

Retrograde labeling in the medulla

Unilateral microinjections of retrobeads solution (20 nl) (n = 4) or fluorogold (5 nl) (n = 2) into the nAmb (Fig. 7A) resulted in bilateral retrograde labeling of cells in the medial NTS with ipsilateral preponderance (Fig. 7B). Retrogradely labeled cells were also observed in the ipsilateral CVLM (Fig. 7C-D) and RVLM (Fig. 7E-F).

Retrograde labeling in the hypothalamus

ARCN (about 2.64 mm long in the rostro-caudal direction) was arbitrarily divided into 3 equal segments: rostral (1.72-2.6 mm caudal to the bregma), middle (2.6-3.48 mm caudal to the bregma) and caudal (3.48-4.36 mm caudal to the bregma) segments. Unilateral microinjections of retrobeads solution (20 nl) or fluorogold (5 nl) into the nAmb resulted in retrograde labeling of cells in the ipsilateral as well as contralateral rostral (Fig. 8A-B) and middle (Fig. 8C-D) segments of the ARCN. PVN (about 1.68 mm long in the rostro-caudal direction) was also arbitrarily divided into 3 equal segments: rostral (0.6-1.16 mm caudal to the bregma), middle (1.16-1.72 mm caudal to the bregma) and caudal (1.72-2.28 mm caudal to the bregma) segments. Unilateral microinjections of retrobeads solution (20 nl) or fluorogold (5 nl) into the nAmb resulted in retrograde bilateral labeling of cells in the rostral (Fig. 9A-B) and middle (Fig. 9C-D) segments of PVN with ipsilateral preponderance. No retrogradely labeled cells were observed in the caudal segments of either ARCN or PVN.
DISCUSSION

The major findings of the present study are as follows: 1) Microinjections of NMDA into the ARCN elicit increases in HR, 2) the tachycardic responses elicited from the ARCN are blocked by prior unilateral and bilateral inhibition of nAmb neurons using microinjections of muscimol, and 3) the increases in HR are attenuated by prior unilateral and bilateral blockade of GABA\textsubscript{A} and glycine receptors in the nAmb.

We have previously reported that the tachycardic responses elicited by chemical stimulation of the ARCN are mediated via stimulation of the sympathetic inputs to the heart as well as inhibition of vagal inputs to the heart (36). The pathways mediating sympathetic stimulation of the heart following the ARCN stimulation have been described in our previous publications (26-27, 36, 41). The tachycardic responses elicited by microinjections of NMDA into the ARCN are not reflex responses to the concomitant falls in BP because similar tachycardic responses are elicited in barodenervated rats (27). As mentioned in the Introduction section, the brain areas and neurotransmitters/receptors in them which mediate the inhibition of the vagal input to the heart following the ARCN stimulation are not known.

It is well established that the vagal input to the heart originates predominantly from the nAmb (13, 43-44). In this study, unilateral and bilateral microinjections of muscimol into the nAmb attenuated the tachycardic responses elicited by the microinjections of NMDA into the ARCN indicating that nAmb is important in mediating these responses. Furthermore, blockade of either GABA\textsubscript{A} receptors by gabazine or glycine receptors by strychnine in the nAmb significantly attenuated the tachycardia elicited from the ARCN suggesting that both GABA and glycine may be released in the nAmb following the chemical stimulation of the ARCN. The attenuation of tachycardic responses elicited by the ARCN stimulation following simultaneous
blockade of GABA<sub>A</sub> and glycine receptors in the nAmb was not significantly greater when
compared to the attenuation of HR responses following the blockade of either GABA<sub>A</sub> or glycine
receptors alone. It is possible, either GABA or glycine is released in the nAmb following the
ARCN stimulation. The situations under which either one of these pathways is activated cannot
be identified based on our results. Thus, inhibition of CVNs by the release of either GABA or
glycine in the nAmb may be the mechanism by which ARCN stimulation decreases the vagal
input to the heart resulting in tachycardia.

The nAmb in the rat consists of a rostral compact portion (nAmbC), a semicompact portion
(nAmbS; located ventral and posterior to the nAmbC) and a loose formation of neurons (nAmbL;
located posterior to nAmbS). The external formation of the nAmb (nAmbE) is located ventral to
the nAmbC and nAmbS at rostral levels and surrounds nAmbL at caudal levels (3, 18). The
precise location and distribution of the CVNs in different regions of the nAmb is not firmly
established. Earlier reports suggested that CVNs are located primarily in the nAmbE at the level
of the CS (3, 18). In recent studies (5, 45), the distribution of CVNs in the different regions of
the nAmb in the rat has been reported as follows: nAmbC (26.2%), nAmbE (19.2%), nAmbL
(10.2%) and nAmbS (8%). The possibility of spread of retrograde tracers into the thoracic
esophagus or other tissues was excluded in these studies (5, 45). Our microinjections of
muscimol, gabazine and strychnine into the nAmb extended from 0.12 mm caudal to 0.64 mm
rostral with reference to the CS. The nAmbC and nAmbS are not present at this rostro-caudal
level of the medulla (38). Therefore, CVNs located in the nAmbE and nAmbL at the levels 0.12
mm caudal to 0.64 mm rostral with reference to the CS were involved in our pharmacological
manipulations. Significant bradycardic responses were elicited by microinjections of L-Glu into
the nAmb at these rostro-caudal medullary levels (32, this study). We placed the microinjections
into the center of the nAmb in order to avoid diffusion of the injectate into the adjacent areas (e.g., caudal ventrolateral medullary depressor area; CVLM) (40, 49). However, the short onset of bradycardia (2 ± 0.1 sec) induced by microinjections of L-Glu into the nAmb indicated that the injected material reached the CVNs located in the nAmbE and nAmbL within seconds.

The pathways from the ARCN to the nAmb that are involved in mediating increases in HR elicited by ARCN stimulation cannot be identified with certainty based on our results. One possibility is that ARCN neurons containing alpha-MSH and/or glutamate (26) may be activated by NMDA, one or both of these excitatory neurotransmitters may be released in the nAmb at the terminals of direct projections to the nAmb, interneurons containing GABA and/or glycine in the nAmb may be activated and the release of GABA and/or glycine in the nAmb may cause inhibition of CVNs with consequent tachycardia. Alternatively, GABA neurons present in the ARCN (20, 26, 41) may be stimulated by NMDA microinjections into the ARCN; GABA may be released at the terminals of direct projections of these neurons in the nAmb causing inhibition of CVNs with subsequent tachycardia. The source of glycinergic input to the CVNs in the nAmb is not known.

Another possibility is that microinjections of NMDA into the ARCN may activate glutamate and/or α-MSH containing cells projecting from the ARCN to the PVN (27), releasing one or both of these neurotransmitters in the PVN which results in the activation of PVN neurons projecting to the nAmb, an excitatory neurotransmitter is released in the nAmb, interneurons containing GABA and/or glycine in this nucleus are stimulated, GABA and/or glycine is released in the nAmb, CVNs are inhibited and HR is increased. Supporting the possible role of this pathway in mediating tachycardia elicited by ARCN stimulation is our previously reported observation that tachycardia elicited by microinjections of NMDA into the ARCN is attenuated.
by the blockade of iGLURS and MC3/4Rs in the PVN (27). Furthermore, blockade of iGLURs in the nAmb significantly attenuated tachycardic responses elicited by microinjections of NMDA into the ARCN (this study).

The implication of pathways mentioned in the preceding paragraphs in mediating the tachycardic responses elicited from the ARCN is supported by our anatomic tracing studies. Direct bilateral projections from the ARCN to the nAmb were identified after microinjecting tracers (green retrobeads and fluorogold) into the nAmb. In agreement with Ciriello et al (12), direct bilateral projections from the PVN to the nAmb were also identified after microinjecting these tracers into the nAmb. Direct projections to the nAmb from the mNTS, CVLM and RVLM were also identified in our study; these observations are consistent with the reports that the CVNs in the nAmb receive inputs from the medullary areas located directly ventral, medial and dorsomedial to the nAmb (16). GABAergic neurons located in the areas ventral and medial to the nAmb may be involved in respiratory modulation of CVNs and cardiorespiratory functions (16).

In the anatomical retrograde tracing techniques used in this study, there is a possibility that axons passing through the injection site in the nAmb, but not terminating there, could have taken up the tracer resulting in the labeling of neurons in the medulla and hypothalamus. This problem was minimized in some experiments by using green retrobeads which are not taken up by fibers of passage (23, 42). However, uptake of retrobeads by fibers of passage damaged during microinjections cannot be definitely excluded.

The CVNs located in the nAmb do not possess inherent pacemaker properties and depend on synaptic neurotransmission for their activation or inhibition (34-35). GABA and glycine have been implicated as major inhibitory neurotransmitters in the nAmb (6, 46-47). Endogenous acetylcholine, norepinephrine, adenosine triphosphate (ATP), and nitric oxide (NO) facilitate
GABAergic and/or glycinergic neurotransmission to CVNs (4, 21, 22, 46-48). Facilitation of GABAergic and/or glycinergic neurotransmission in the nAmb has been implicated in some physiological and pathophysiological conditions. For example, acetylcholine-induced facilitation of GABAergic and/or glycinergic neurotransmission to CVNs in the nAmb may be involved in the generation of respiratory sinus arrhythmias (48). Activation of alpha 1-adrenergic receptors by norepinephrine facilitates GABAergic and glycinergic neurotransmission in the nAmb reducing the activity of CVNs which may explain tachycardia observed in norepinephrine-dependent behavioral arousal (4). One of the mechanisms by which CVNs are excited during hypoxia may be disinhibition of these neurons via withdrawal of GABAergic and glycinergic neurotransmission (15). The inhibitory pathway to the nAmb identified in our study may play a role in stress-induced tachycardia. In this context, it may be noted that the ARCN is one of the brain sites for the location of neurons that are activated during stress (31, 37).

There are some technical issues that are relevant to this study and need some discussion. The onset, peak and duration of NMDA effects in the ARCN are relatively long. These longer time intervals cannot be ascribed to diffusion of the injected material to areas located adjacent to the ARCN because the volume of microinjection in our study is small. We have previously shown that microinjections of NMDA (30-50 nl) into the ARCN do not diffuse to the dorsal median nucleus of the hypothalamus which is located immediately dorsal to the ARCN (36). Longer onset and peak times of the responses to NMDA microinjections into the ARCN may be due to the fact that ARCN consists of heterogeneous populations of neurons and longer times may be needed for NMDA to reach the target neurons. Possible involvement of relay neurons (e.g., PVN neurons) may also contribute to these longer time intervals. NMDA is a non-metabolizable analogue of D-aspartic acid, it may remain at the injection site for a longer time,
thus increasing the duration of NMDA effects. At the beginning of each experiment, the ARCN was functionally identified by microinjections of NMDA instead of L-Glu because very high doses of L-Glu are needed for stimulation of this nucleus (36). On the other hand, nAmb was functionally identified by microinjections of L-Glu consistent with our previous reports (6, 8-10). Cardiovascular responses elicited from the ARCN or nAmb were not due to the distortion of the brain tissue because microinjections of aCSF into these nuclei elicited no cardiovascular responses.

Perspectives

In the present paper, we have demonstrated that ARCN stimulation elicits increase in HR which is mediated via GABA_A and glycine receptors in the nAmb. The ARCN is located close to the median eminence which has a high capillary density and high capillary blood flow and lacks blood-brain barrier (17). Because of these anatomic features, the ARCN may be accessible to physiologically active substances circulating in the blood. The results of the present study provide the groundwork for testing the role of ARCN in mediating HR and other cardiovascular responses to circulating substances that are released during stress.
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Fig. 1. Attenuation of tachycardia elicited from the ARCN by inhibition of neurons in the nAmb.

A: Increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN (n = 9; baseline value of HR: 387.3 ± 14.4 bpm). B: In the same group of rats, increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN was significantly attenuated by prior unilateral microinjections of muscimol (1 mM) into the ipsilateral nAmb (baseline value: 388.9 ± 12.2 bpm). C: In another group of rats (n = 6), increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN (baseline value: 363.2 ± 11.3 bpm). D: In the same rats, increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN was significantly attenuated by prior bilateral microinjections of muscimol (1 mM) into the nAmb (baseline value: 368.2 ± 11.6 bpm). E-J: Tracings from one experiment; top trace: MAP (mmHg); bottom trace: HR (bpm). E: Microinjection of NMDA (10 mM) into the ARCN elicited decrease in MAP and increase in HR. F and G: Twenty min later, nAmb was identified bilaterally with microinjections of L-Glu (5 mM); a decrease in HR was elicited with no changes in MAP. H and I: After 20 min, muscimol (1 mM) was bilaterally microinjected into the nAmb; an increase in HR was elicited. J: Five min later, tachycardia elicited from the ARCN was significantly attenuated (compare with E). Abbreviations: ARCN: arcuate nucleus; HR: heart rate (bpm); L-Glu: L-glutamate; Lt: left; MAP: mean arterial pressure; Mus: muscimol; nAmb: nucleus ambiguus; NMDA: N-methyl-D-aspartic acid; Rt: right. In this and other figures, asterisks represent p values as follows: * = p < 0.05, ** = p < 0.01, *** = p < 0.001 and **** = p < 0.0001.
Fig. 2. Attenuation of tachycardia elicited from the ARCN by GABA<sub>A</sub> receptor blockade in the nAmb. A: Increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN (n = 9; baseline value of HR: 370.4 ± 15.5 bpm). B: In the same group of rats, increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN was significantly attenuated by prior unilateral microinjections of gabazine (0.01 mM) into the ipsilateral nAmb (baseline value: 346.1 ± 17.2 bpm). C: In another group of rats (n = 8), increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN (baseline value: 355.3 ± 15.5 bpm). D: In the same rats, increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN was significantly attenuated by prior bilateral microinjections of gabazine (0.01 mM) into the nAmb (baseline value: 330.5 ± 14.7 bpm). E-J: Tracings from one experiment. E: Microinjection of NMDA (10 mM) into the ARCN elicited decrease in MAP and increase in HR. F and G: Twenty min later, nAmb was identified bilaterally with microinjections of L-Glu (5 mM); a decrease in HR was elicited with no changes in MAP. H and I: After 20 min, gabazine (0.01 mM) was bilaterally microinjected into the nAmb; no significant change in HR was elicited by this dose of gabazine. J: Five min later, tachycardia elicited from the ARCN was significantly attenuated (compare with E). Labels for tracings and abbreviations are same as in Fig. 1; Gabaz: gabazine.
Fig. 3. Attenuation of tachycardia elicited from the ARCN by glycine receptor blockade in the nAmb. A: Increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN (n = 5; baseline value of HR: 341.4 ± 5.9 bpm). B: In the same group of rats, increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN was significantly attenuated by prior unilateral microinjections of strychnine (0.5 mM) into the ipsilateral nAmb (baseline value: 345 ± 12.2 bpm). C: In another group of rats (n = 5), increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN (baseline value: 349.2 ± 13 bpm). D: In the same rats, increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN was significantly attenuated by prior bilateral microinjections of strychnine (0.5 mM) into the nAmb (baseline value: 329.4 ± 16.1 bpm). E-J: Tracings from one experiment. E: Microinjection of NMDA (10 mM) into the ARCN elicited decrease in MAP and increase in HR. F and G: Twenty min later, nAmb was identified bilaterally with microinjections of L-Glu (5 mM); a decrease in HR was elicited with no changes in MAP. H and I: After 20 min, strychnine (0.5 mM) was bilaterally microinjected into the nAmb; no significant change in HR was elicited by this dose of strychnine. J: Five min later, tachycardia elicited from the ARCN was significantly attenuated (compare with E). Labels for tracings and abbreviations are same as in Fig. 1. Strych: strychnine.
Fig. 4. A-B: Attenuation of tachycardia elicited from the ARCN by combined microinjections of GABA<sub>A</sub> and glycine receptor blockade in the nAmb. A: Increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN (n = 5; baseline value: 397.2 ± 4.2 bpm). B: In the same rats, increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN was significantly attenuated by prior unilateral combined microinjections of gabazine (0.01 mM) and strychnine (0.5 mM) into the ipsilateral nAmb (baseline value: 358.8 ± 5.7 bpm).

C-D: Attenuation of tachycardia elicited from the ARCN by the blockade of iGLURs in the nAmb. C: Increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN (n = 7; baseline value: 361.7 ± 7.4 bpm). D: In the same rats, increase in HR elicited by unilateral microinjections of NMDA (10 mM) into the ARCN was significantly attenuated by prior unilateral microinjections of D-AP7 (5 mM) and NBQX (2 mM) into the nAmb (baseline value: 367.9 ± 10 bpm).

Fig. 5. Histological identification of microinjection sites in ARCN. A: A coronal section at a level 2.28 mm caudal to the bregma showing a microinjection site in the ARCN marked with green retrobeads solution (arrow); the center of the spot was 0.35 mm lateral to the midline and 9.7 mm deep from the dura (Bar = 500 µm). B, C, D, E, F and G: Drawing of coronal sections 3.72, 3.48, 3.12, 2.76, 2.28 and 1.92 mm caudal to the bregma showing the ARCN microinjection sites as dark spots; each spot represents a site in one animal. The microinjection sites were located in the ARCN, 0.1-0.6 mm lateral to the midline and 9.6-10.1 mm deep from the dura. 3V: 3rd ventricle; ARCN: hypothalamic arcuate nucleus; f: fornix. Bar = 1 mm.
Fig. 6. Histological identification of microinjection sites in nAmb. A: A coronal section at a level 0.24 mm rostral with reference to the CS showing a microinjection site in nAmb on the right side marked with green retrobeads solution (arrow); the center of the spot was 1.9 mm lateral to the midline and 2.1 mm deep from the dura. B-H: Drawings of coronal sections at different levels of the medulla showing the nAmb microinjection sites as dark spots; each spot represents one animal. B, C, D, E, and F: 0.64, 0.48, 0.36, 0.24 and 0.12 mm rostral to CS. G: at the level of the CS. H: 0.12 mm caudal to the CS. AP: area postrema; CC: central canal; CS: calamus scriptorius; nAmb: nucleus ambiguus; py: pyramid; 10N: dorsal motor nucleus of vagus; 12N: hypoglossal nucleus. Bar = 1 mm.

Fig. 7. Retrograde tracing of medullary projections to the nAmb. A: A coronal section at a level 0.20 mm rostral to the CS showing microinjection site (arrow) in the nucleus ambiguus (nAmb) marked with fluorogold and retrograde labeling in the NTS (box; scale bar = 1 mm). B: Higher magnification of the boxed area in panel A showing retrograde labeling of NTS neurons (bar = 200 µm). C: A coronal section at a level 1.32 mm rostral to the CS showing retrograde labeling of fluorogold in the CVLM (box; bar = 1 mm). D: Magnified boxed area in panel C showing retrograde labeling of CVLM neurons (bar = 200 µm). E: A coronal section at a level 1.92 mm rostral to the CS showing retrograde labeling of fluorogold in the RVLM (box; bar = 1 mm). F: Higher magnification of the boxed area in panel E showing retrogradely labeled RVLM neurons (bar = 200 µm).
Fig. 8. Retrograde tracing of ARC N projections to the nAmb. A: A coronal section at a level 1.80 mm caudal to the bregma showing bilateral retrograde labeling of the rostral ARC N (box; bar = 200 μm) after a microinjection of fluorogold into the nAmb. B: Higher magnification of the boxed area in panel A showing retrogradely labeled ARC N cells (bar = 100 μm). C: A coronal section at a level 3.36 mm caudal to bregma showing bilateral labeling of cells in the middle ARC N (bar = 200 μm). D: Magnified boxed area in the panel C showing retrograde labeling of ARC N neurons (bar = 100 μm). Abbreviations: ARC N: hypothalamic arcuate nucleus; 3V: 3rd ventricle.

Fig. 9. Retrograde tracing of PV N projections to the nAmb. A: A coronal section at a level 1.08 mm caudal to bregma showing retrogradely labeled cells in the ipsilateral rostral PV N (bar = 200 μm) after a microinjection of fluorogold into the nAmb. B: Magnification of the boxed area in the panel A showing retrograde labeled cells in the ipsilateral rostral PV N (bar = 100 μm). C: A coronal section at a level 1.32 mm caudal to bregma showing retrogradely labeled cells in the middle PV N (bar = 200 μm). D: Magnified boxed area in the panel C showing retrogradely labeled cells in the ipsilateral middle PV N (bar = 100 μm).
Fig. 1

**A** and **B**: Increase in HR (bpm) after unilateral Mus nAmb infusion. **C** and **D**: Increase in HR (bpm) after bilateral Mus nAmb infusion.

**E** and **F**: MAP (mmHg) and HR (bpm) recordings after ARCN (NMDA: 10 mM) and Rt. & Lt. nAmb (L-Glu: 5 mM) infusion.

**G** and **H**: MAP (mmHg) and HR (bpm) recordings after Rt. & Lt. nAmb (Muscimol: 1 mM) and ARCN (NMDA: 10 mM) infusion.

*Note: Significance levels indicated by *****.
Fig. 2

**A** and **B**: Increase in HR (bpm) for Gabazine (nAmb) Unilateral.

**C** and **D**: Increase in HR (bpm) for Gabazine (nAmb) Bilateral.

**E** and **F**: MAP (mmHg) and HR (bpm) for ARCn.

**G**: Rt. & Lt. nAmb (NMDA: 10 mM).

**H** and **I**: MAP (mmHg) and HR (bpm) for ARCn (Gabazine: 0.01 mM).

**J**: 2 min

**N** and **N**: NMDA into ARCn.
Fig. 3

Rt. & Lt. nAmb (Strychnine: 0.5 mM)  
ARCN (NMDA: 10 mM)
Fig. 4

**Increase in HR (bpm)**

- **Gabaz + Strych**: n = 5
  - A
  - B
  - ***

- **D-AP7 + NBQX**: n = 7
  - C
  - D
  - *

NMDA into ARC N
Fig. 6
Fig. 9