ONLINE SUPPLEMENT

Tonic GABAergic Activity in the Hypothalamic Arcuate Nucleus Is Attenuated in the Spontaneously Hypertensive Rat

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Short title: Arcuate nucleus: role in hypertension

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Methods

Ethics Statement
Experiments were done according to the NIH guide for “The Care and Use of Laboratory Animals, 7th Edition, 1996” and the protocols for the experiments were approved by the Institutional Animal Care and Use Committee of this institution.

General Procedures and Anesthesia
The animals were housed in the animal care facility of this institution under controlled conditions with a 12:12-hour light-dark cycle. Food and water were allowed to the animals ad libitum. The rats were anesthetized with inhalation of isoflurane (2–3% in 100% oxygen). A tracheostomy was performed and the rats were artificially ventilated using a rodent ventilator (model 683; Harvard Apparatus, Holliston, MA, USA). One of the femoral arteries was cannulated for monitoring BP. Mean arterial pressure (MAP) and HR were derived electronically from BP waves. One of the femoral veins was cannulated and urethane (1.2–1.4 g/kg) was injected intravenously in 8-9 aliquots at 2-min intervals (total volume of the anesthetic solution was 0.4–0.45 ml injected over a period of about 16–18 min). Isoflurane inhalation was terminated as soon as urethane administration was completed. Rectal temperature was maintained at 37 ± 0.5°C using a temperature controller (model TCAT-2AC, Physitemp Instruments, Clifton, NJ, USA). All of the tracings were stored on a computer hard drive using a data acquisition system obtained from Cambridge Electronic Design Ltd (CED; Cambridge, UK). At the end of the experiment, the rats were deeply anesthetized with a high dose of urethane (2 g/kg, i.v.), a pneumothorax was produced by an incision in one of the intercostal muscles and cessation of heart beat indicated that euthanasia was complete.

Microinjections into the hypothalamic arcuate nucleus (ARCN)
The rats were placed in a prone position in a stereotaxic instrument (David Kopf Instruments, Tajunga, CA, USA) with bite bar 3.3 mm below the interaural line. The bregma was visually identified and a small hole was drilled in the parietal bone. Multi-barreled glass-micropipettes (tip size 20–40 μm) were used for microinjections. For microinjection into the ARCN, the micropipettes were inserted into the brain perpendicularly. The coordinates for microinjections into the ARCN were: 1.9-4.1 mm caudal to the bregma, 0.2–0.4 mm lateral to the midline, and 9.6–9.9 mm deep from the dura.

Histology
At the end of the experiment, diluted green retrobeads IX (1: 50) were microinjected into the ARCN as a marker to confirm microinjection sites. The animals were perfused and fixed with 2% paraformaldehyde and serial sections of the hypothalamus were cut (40 μm) in a vibratome and mounted on subbed slides, covered with Citifluor mountant medium (Ted Pella Inc., Redding, CA, USA) and coverslipped. The microinjection sites were identified under a microscope (model AX70, Olympus Provis, Middlebush, NJ, USA) and the sections were photographed (Neurolucida software, version 7.5, MicroBrightField Inc., Williston, VT, USA) and compared with a standard atlas.1
**Greater splanchnic nerve recording**
The greater splanchnic nerve (GSN) was identified under an operating microscope (OPMI-1, Carl Zeiss, Thornwood, NY, USA) using a retroperitoneal approach. The GSN was sectioned at its junction with the celiac ganglion, a small segment was desheathed and its activity was recorded using a bipolar silver wire hook electrode. The activity of whole nerve (GSNA) was amplified (×10,000–20,000), filtered (100–5000 Hz), digitized and stored on a computer hard drive. The digitized signals were full-wave rectified and integrated over consecutive 1 sec intervals using Spike 2 program (CED, UK). At the end of the experiment, the nerve was sectioned centrally and the remaining activity was considered to be the noise level which was subtracted from the GSNA amplitude.

**Barodenervation**
The carotid sinus, aortic depressor and recurrent laryngeal nerves were identified under an operating microscope and sectioned bilaterally. Lack of bradycardia and inhibition of GSNA in response to intravenous bolus injections of phenylephrine (10 µg/kg) indicated that the barodenervation was complete.

**Drugs and Chemicals**
All of the solutions for the microinjections were freshly prepared in artificial cerebrospinal fluid (aCSF, pH 7.4). The composition of aCSF was as follows: NaCl (128 mM), KCl (3 mM), CaCl₂ (1.2 mM), MgCl₂ (0.8 mM), dextrose (3.4 mM) and HEPES (5 mM). Where applicable, the concentration of drugs refers to their salts. The vendors for different drugs and chemicals were as follows: CGP52432 (R&D Systems, Minneapolis, MN, USA), isoflurane (Piramal Critical Care, Bethlehem, PA, USA), green retrobeads IX (Lumafluor Inc., Durham, NC, USA). All other drugs and chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA).

**Statistical Analyses**
The means and standard error of the means (S.E.M.) were calculated for maximum changes in MAP and HR in response to microinjections of different drugs into the ARCN. One-way analysis of variance (ANOVA) followed by Tukey-Kramer's multiple test was used for determination of concentration-response of gabazine and NMDA responses in the ARCN. Student’s unpaired t-test was used for comparison of the following responses: increases in MAP and HR induced by the microinjections of gabazine, CGP52432 and NMDA into the ARCN in different groups of both Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). For analyses of the GSNA, the integrated signals obtained just before the microinjections of gabazine, CGP52432 and NMDA into the ARCN were averaged over a period of 60 sec. When the responses to these treatments were maximal, the integrated signals were averaged over a period of 60–90 sec. The percentage changes in GSNA elicited by these treatments were calculated and compared between different groups of rats by using Student's unpaired t-test. In all cases, the differences were considered significant at P < 0.05.
Results

**GABAB Receptor Blockade in the ARCN**
The concentration of CGP52432 (20 mmol/L) used in these experiments, was selected from published literature. In all groups of WKY and SHR, CGP52432 microinjected in the ARCN elicited increases in MAP and GSNA, but no change in HR (Table S1). There was no significant difference in MAP and GSNA responses elicited by CGP52432 between WKY and SHR. Lower concentration (e.g., 10 mmol/L) of CGP52432 did not elicit any significant change in MAP. Concentrations of CGP52432 higher than 20 mmol/L could not be used because of the lack of solubility of this antagonist in aCSF at these concentrations.

The onset, peak, and duration of responses to microinjections of CGP52432 into the ARCN were 10-25 seconds, 3-7 minutes and 20-35 minutes, respectively. Cardiovascular responses elicited by microinjections of CGP52432 (20 mmol/L) into the ARCN were smaller than those elicited by gabazine (2 mmol/L) (Tables 1 and S1).

**References**
Figure S1. Histological Identification of Microinjection Sites in the ARCN.

A: A typical microinjection site in the ARCN marked with green retrobeads IX (20 nL) in SHR. B-D: composite diagrams of ARCN sections at selected levels 2.40 mm (rostral region), 3.24 mm (middle region) and 3.84 mm (caudal region) showing microinjection sites in WKY (open squares) and SHR (dark triangles); the coordinates mentioned below each drawing are caudal to the bregma. In both WKY and SHR, microinjections were made unilaterally (left or right side) as well as bilaterally. In the diagrams, for simplification, microinjection sites in WKY are shown at the left side and those in SHR are shown at right side. Bar in panel A = 500 μm. DMN: the hypothalamic dorsomedial nucleus; PH: posterior hypothalamic nucleus; PMV: ventral premammillary nucleus; VMN: the hypothalamic ventromedial nucleus; 3V: the third ventricle.
Table S1. Responses to Microinjections of CGP52432 into the ARCN.

<table>
<thead>
<tr>
<th>Concentration (mmol/L)</th>
<th>n</th>
<th>Increase in MAP (mmHg)</th>
<th>Increase in GSNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WKY</td>
<td>SHR</td>
<td>WKY</td>
</tr>
<tr>
<td>Baro-intact rats: unilateral microinjections</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>20</td>
<td>6</td>
<td>10.1 ± 1.3</td>
<td>8.6 ± 0.9</td>
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<tr>
<td>20</td>
<td>5</td>
<td>9.0 ± 0.4</td>
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<td>Barodenervated rats: unilateral microinjections</td>
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<td>20</td>
<td>5</td>
<td>11.2 ± 1.4</td>
<td>10.0 ± 1.2</td>
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</table>

The values shown for WKY and SHR were not statistically different. n: number of rats.