INCREASED EXPRESSION OF AT2 RECEPTORS IN THE SOLITARY-VAGAL COMPLEX BLUNTS RENOVASCULAR HYPERTENSION

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Short title: NTS/DMV AT2R and blood pressure in 2K1C rats

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Methods

Animals

Male Holtzman rats and male Sprague Dawley (SD) rats (150 - 180 g) were used for these experiments. Rats were kept on a 12-hour light/dark cycle in climate-controlled rooms. Rat chow and water were provided *ad libitum*. The Ethics Committee for Animal Care and Use (CEUA) of the School of Dentistry of Araraquara, São Paulo State University approved all experimental protocols used for Holtzman rats (Proc. CEUA 07/2011), and the University of Florida Institutional Animal Care and Use Committee approved all experimental protocols with the SD rats. In addition, the principles governing the care and treatment of animals, as stated in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (eighth edition, 2011) and adopted by the American Physiological Society, were followed at all times during this study.

Renovascular hypertension model and implantation of telemetry transmitters

Male Holtzman rats were anesthetized with ketamine [80 mg/kg of body weight (b. wt.)] combined with xylazine (7 mg/kg of b. wt.), and underwent two surgeries. First, the left renal artery was partially obstructed using a silver clip of 0.2 mm width. This occlusion significantly reduces renal blood flow and elicits hypertension.[1] Normotensive animals were submitted to the same surgical procedure without partial renal artery occlusion (sham surgery). We will refer Holtzman rats with sham surgery as normotensive (NT) rats. Immediately following implantation of the silver clip or sham surgery, rats were implanted with telemetry transducers (Data Sciences International; TA11PAC40) in the abdominal aorta as described previously, to record arterial pressure and heart rate.[2,3] Following the surgeries animals received a prophylactic dose of penicillin (50,000 IU, intramuscularly) and a dose of the anti–inflammatory ketoprofen (1 mg/kg of b.w, subcutaneously).

In vivo gene transfer in the solitary-vagal complex

Holtzman rats were anesthetized with ketamine and xylazine as described above and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A partial craniotomy of the occipital bone was performed, and the dorsal surface of the brainstem was exposed. AAV2-CBA-eGFP or AAV2-CBA-AT2R were microinjected at 5 different sites along the solitary-vagal complex (comprised of the NTS and DMV or NTS/DMV). Each microinjection consisted of 150 nL vector/site (eGFP,$1.5x10^{12}$ genome copies and AT2R, $3.6x10^{11}$ genome copies [gc]/injection) and was performed as follows: at the level of the *calamus scripitorius* and 0.5 mm rostral and 0.4 mm caudal to it; 0.2-0.5 mm lateral from the midline and 0.4 mm ventral to the dorsal surface of the medulla . Each injection was made over a period of 1 min. A prophylactic dose of penicillin (50,000 IU, intramuscularly) and of the anti–inflammatory ketoprofen (1 mg/kg of b.wt, subcutaneously) were given post surgically. Identical microinjections of AAV2-CBA-eGFP or AAV2-CBA-AT2R were made in SD rats, the only differences being that anesthesia was induced using 100% O_2/4% isoflurane, and was maintained throughout the surgeries by the administration of 100% O_2/2% isoflurane, and that buprenorphine (0.05 mg/kg, s.c., Hospira Inc., Lake Forest, IL, USA) was administered to rats immediately following the survival surgeries. During the surgeries/procedures, the level of anesthesia was monitored by checking the eye blink reflex and a reaction to paw pinch, and was adjusted if necessary. AAV2-CBA-eGFP or AAV2-CBA-AT2R

were constructed as described previously,[4,5] and these vectors elicit gene transduction primarily in neurons of all different phenotypes.[6]

Baroreflex Function

On the day before the experiment, rats were anesthetized with ketamine and xylazine as described above, and a polyethylene tube (PE-10 connected to a PE-50) was inserted into the abdominal aorta through the femoral artery and another catheter was implanted into the femoral vein. Arterial and venous catheters were tunneled subcutaneously and exposed on the back of the rat. To record pulsatile arterial pressure, MAP and HR in conscious unrestrained, freely moving animals, the arterial catheter was connected to a Statham Gould (P23 Db; El Segundo, CA, USA) pressure transducer coupled to a pre-amplifier (model ETH-200 Bridge Bio Amplifier, Chicago, IL, USA) that was connected to a Powerlab computer data acquisition system (model Powerlab 16SP, ADInstruments, Colorado Springs CO, USA). After a baseline period of cardiovascular recordings, rats received iv injections of phenylephrine (5 µg/kg, b.wt.) or sodium nitroprusside (SNP; 30 µg/kg, b.wt.) to test the HR reflex responses to pressor and depressor stimuli, respectively. We analyzed the one second mean HR values in response to 10 mmHg incremental changes in MAP, starting in 5 mmHg up to a maximal change of 35 mmHg. The values were plotted, a linear regression was performed for each animal, and the slope of each linear regression was used to calculate the differences between groups.

Euthanasia

Rats were euthanized by placing them under deep anesthesia with either isoflurane (5% in 100% O_2) or sodium thiopental (70 mg/kg of b. wt, ip), followed by either decapitation or transcardial perfusion with chilled 0.9% saline followed by 4% paraformaldehyde, depending on the protocol.

eGFP fluorescence in the NTS

Animals were perfused transcardially, brainstems were removed, frozen and cut in coronal sections (30-µm thickness) on a cryostat (Leica, CM1850 UV, Wetzlar, Hesse, Germany). The sections were visualized using a Leica fluorescence microscope (Leica, DM5500 B, Wetzlar, Hesse, Germany) with the appropriate filter to visualize the eGFP expression. We used the area postrema (AP) and central canal as anatomical landmarks to define the rostro-caudal and dorso-ventral levels of the NTS/DMV; the intermediate NTS (iNTS) is located at AP level and commissural NTS (cNTS) is located caudal to the AP. The NTS/DMV complex is dorsal to the central canal.

Kidney analysis

At the end of the experiments, rats were weighed and under deep anesthesia as described above, the kidneys were removed and weighed to confirm atrophy in the clipped kidney and hypertrophy in the contralateral kidney.[7] After kidney removal, some animals were decapitated for brain microdissection (see below).

qRT–PCR analyses in the NTS/DMV

Animals were decapitated, the brains were removed and the NTS was collected by micropunch with the aid of a surgical microscope using the obex as a reference site. AT2R, AT1R and ACE2 mRNA levels within the NTS were analyzed by qRT–PCR as

detailed previously [2] and were expressed using the ΔCT method normalized to 18S mRNA levels.

AT2R autoradiography in the NTS/DMV

Animals were perfused transcardially with chilled phosphate buffered saline. The brains were removed quickly, wrapped in aluminum foil, and frozen at -80 °C until sectioned. Coronal sections (20-µm thickness) were cut from rat brains using a cryostat (Leica, CM1850 UV, Wetzlar, Hesse, Germany) and thaw-mounted onto gelatin-coated slides. The sections were allowed to dry at room temperature and then stored at -80°C until the time of autoradiography. The frozen sections were thawed and in vitro receptor autoradiography was performed using an established protocol. [8] Briefly, brain sections were incubated with 500 pM ^{125}I-labeled Sar^{1}, Ile^{8} Angiotensin II in the presence or absence of 10 µM PD123319 to block AT_2 receptors, 10 µM losartan to block AT_1 receptors, or 3 µM Angiotensin II to establish non-specific binding. X-ray films exposed to ^{125}I-labeled sections were scanned and analyzed using MCID (Interfocus Imaging Ltd.). Brain regions of interest were manually circumscribed using the rat brain atlas of Paxinos and Watson, 7th edition (2014) as a guide. MCID automatically calculated average relative optical density values of circumscribed areas. Specific binding to the AT_1 or AT_2 receptors was defined as the difference between nonspecific binding and binding in the presence of PD123319 or losartan, respectively.

Cardiovascular variability analysis

The pulse interval (PI) and systolic arterial pressure (SAP) variability analyses were performed using a custom computer software (CardioSeries v2.4 – http://www.danielpenteado.com), as described previously. [9-11] Brifely, beat-by-beat series obtained from pulsatile arterial pressure recordings were converted to data points every 100 ms using cubic spline interpolation (10 Hz). The interpolated series was divided into half-overlapping sequential sets of 512 data points (51.2 s). Before calculation of the spectral power density, segments were visually inspected and non-stationary data were not taken into consideration. A Hanning window was used to attenuate side effects and the spectrum was computed using a direct FFT algorithm for discrete time series. The spectra were integrated in low-frequency (LF; 0.2–0.75 Hz) and high-frequency (HF; 0.75–3 Hz) bands, and results are expressed in absolute for SAP (mmHg2) and normalized units (nu) for PI. The normalized values were achieved by calculating the percentage of LF and HF power with regard to the total power of spectrum minus very low frequency band (VLF; <0.2 Hz) power. [12,13] To assess the sympathovagal balance, LF/HF ratio of PI variability was calculated.[14]

Data analysis

All data are expressed as means ± SEM. Different statistical tests were applied for group comparisons depending on the experimental design. One or two-way ANOVA was used and both were followed by Sidak's multiple comparisons (for autoradiography data) or Student Newman-Keuls (for all other experiments) *post hoc* tests to compare individual means. In all tests differences were considered significant at $p<0.05$.

References

1. Ploth DW, Roy RN, Huang WC, Navar LG. Impaired renal blood flow and cortical pressure autoregulation in contralateral kidneys of Goldblatt hypertensive rats. *Hypertension*. 1981; 3:67-74.
2. Li H, Gao Y, Freire CD, Raizada MK, Toney GM, Sumners C. Macrophage migration inhibitory factor in the PVN attenuates the central pressor and dipsogenic actions of angiotensin II. *FASEB J*. 2006; 20:1748-1750.
3. Freiria-Oliveira AH, Blanch GT, Li H, Colombari E, Colombari DSA, Sumners C. Macrophage migration inhibitory factor in the nucleus of solitary tract decreases blood pressure in SHRs. *Cardiovasc Res*. 2013; 97:153-160.
4. Li HW, Gao YX, Raizada MK, Sumners C. Intronic enhancement of angiotensin II type 2 receptor transgene expression in vitro and in vivo. *Biochem Biophys Res Commun*. 2005; 336:29-35.
5. Li HW, Gao YX, Matsuura T, Martynyuk A, Raizada MK, Sumners C. Adenoviral-mediated neuron specific transduction of angiotensin II type 2 receptors. *Regul Pept*. 2005; 126:213-222.
6. Zhang Y, Gao Y, Speth RC, Jiang N, Mao Y, Sumners C, Li H. Adenoviral and adeno-associated viral vectors-mediated neuronal gene transfer to cardiovascular control regions of the rat brain. *Int J Med Sci*. 2013; 10:607-616.
7. Smith SH, Bishop SP. Selection criteria for drug-treated animals in two-kidney, one clip renal hypertension. *Hypertension*. 1986; 8:700-705.
8. Speth RC, Barry WT, Smith MS, Grove KL. A comparison of brain angiotensin II receptors during lactation and diestrus of the estrous cycle in the rat. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 1999; 277:R904-R909.
9. Tezini GCSV, Dias DPM, Souza HCD. Aerobic physical training has little effect on cardiovascular autonomic control in aging rats subjected to early menopause. *Experimental Gerontology*. 2013; 48:147-153.
10. Pires W, Wanner SP, Lima MR, Fonseca IA, Fumega U, Haibara AS, Coimbra CC, Lima NR. Physical exercise performance in temperate and warm environments is decreased by an impaired arterial baroreflex. *PLoS One*. 2013; 8:e72005.
11. Sabino JP, da Silva CA, de Melo RF, Fazan R, Jr., Salgado HC. The treatment with pyridostigmine improves the cardiocirculatory function in rats with chronic heart failure. *Auton Neurosci*. 2013; 173:58-64.
12. Billman GE. Heart rate variability - a historical perspective. *Front Physiol*. 2011; 2:86.
13. van de Borne P, Montano N, Pagani M, Oren R, Somers VK. Absence of low-frequency variability of sympathetic nerve activity in severe heart failure. *Circulation*. 1997; 95:1449-1454.
14. Montano N, Ruscone TG, Porta A, Lombardi F, Pagani M, Malliani A. Power spectrum analysis of heart rate variability to assess the changes in sympathovagal balance during graded orthostatic tilt. *Circulation*. 1994; 90:1826-1831.

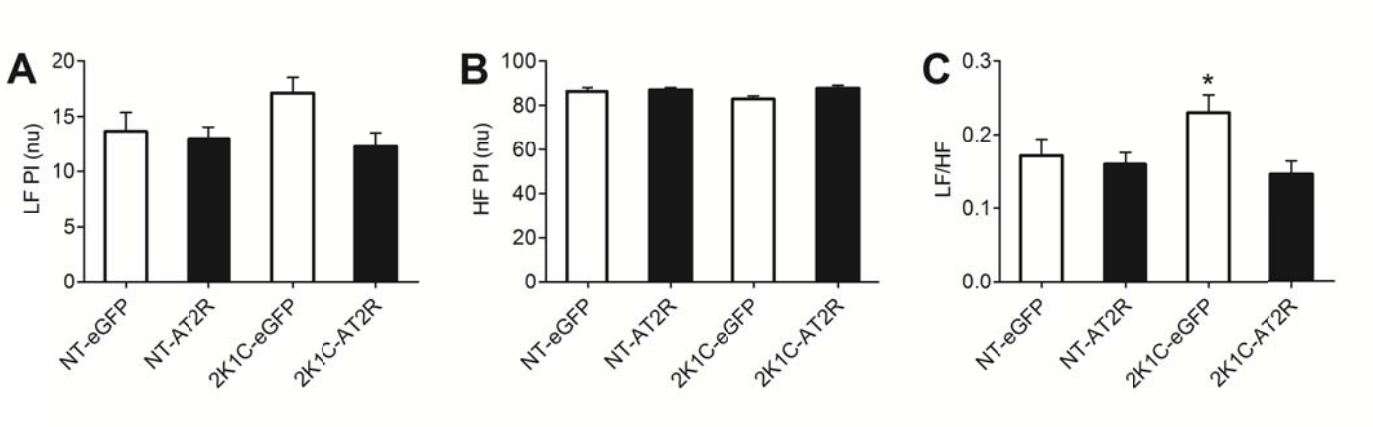

Figure S1: Normotensive (NT) or 2K1C rats received intra-NTS/DMV injections of AAV2-CBA-eGFP or AAV2-CBA-AT2R followed by analysis of the PI variability. Power of the low and high frequency bands (LF, panel A; HF, panel B) of pulse interval (PI), and LF/HF ratio (panel C). * $p < 0.05$ vs. all groups.