ONLINE SUPPLEMENT

CIRCULATING ANGIOTENSIN II GAINS ACCESS TO THE HYPOTHALAMUS AND BRAINSTEM DURING HYPERTENSION, VIA BREAKDOWN OF THE BLOOD BRAIN BARRIER

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Expanded Methods

Animals and experimental groups

Spontaneously Hypertensive (SHR), Wistar Kyoto (WKY) and Wistar rats were purchased from Harlan Laboratories (IN), maintained under a 12h: 12h light-dark cycle and given free access to food and water unless under anti-hypertensive treatment. All procedures were carried out in agreement with the Georgia Regents University Institutional Animal Care and Use Committee Guidelines. SHR and WKY animals were divided in 4 groups as follows: 1) SHR; 2) WKY; 3) SHR + losartan treatment (20mg/kg/day in drinking water; Sigma-aldrich, MO) and 4) SHR + hydralazin treatment (10mg/kg/day in drinking water; Sigma-aldrich, MO). Treatment with Losartan started when animals were 5 to 6-week-old, and lasted for 7 weeks. Hydralazine treatment started in 8 to 9-week-old animals, and lasted for 4 weeks. All final experiments were performed in 12 to 13-week-old rats. In another set of experiments, Wistar rats were divided in 1) renovascular hypertensive induced animals (RVH) or 2) Sham-operated animals. Experiments on these groups were performed 6 weeks after surgical procedure.

Induction of 2 kidney 1 clip hypertension (renovascular hypertension – RVH)

RVH was induced as previously described1-3. Briefly, 150-180 male Wistar rats were anesthetized induced and maintained with 3% isoflurane and an abdominal incision to expose the left renal artery was performed. A silver clip of 0.2-mm width was placed around the left renal artery to partially obstructed renal blood flow. Sham-operated groups underwent the same surgical procedure but no clip obstruction was applied. Post-operative care included proper management of associated pain (Buprenorphine, 0.25 mg/kg, subcutaneous). Blood pressure was measured in conscious rats using a tail cuff plethysmography method 5 weeks after surgery. Ten consecutive measurements were taken, and an average was reported. Final experiments were performed 6 weeks after the surgical procedure.
Blood pressure measurements

Systolic blood pressure (SBP) was measured using a non-invasive tail cuff acquisition method (coda 6; Kent Scientific Corporation, CT). Animals were placed into a restrainer and let sit in the holders over a heating pad for at least 5 minutes to allow rats to acclimate and the optimal body temperature (~33-35°C) to be reached. All animals were first acclimated to the SBP measurements for 3 days before the final acquisition. In the 4th day, five acclimation cycles were performed before the fifteen measurement cycles, which were averaged to obtain the SBP reported of each animal. Measurements were taken before and after drug treatments.

Intravascular injections of fluorescent dextrans

After functional measurements, rats were anesthetized (Ketamine/Xylazine cocktail – 60 mg and 8 mg/ml respectively) and a non occluding catheter filled with fluorescent dye was inserted into the left internal carotid artery as previously described4, 5. The fluorescent dextrans Rhodamine 70 kDa (RHO70; 10mg/mL, 2.86μl/g/each; Sigma-aldrich, MO) and FITC 10 kDa (FITC10, 10mg/mL, 2.86μl/g/each; Sigma-aldrich, MO), or the fluorescently-labeled angiotensin II (AngIIfluo, 1μmol/L, 2.86μl/g/each; Anaspec, CA), were then injected at a slow rate (~300 μl/60 s). The fluorescent dyes were allowed to circulate for 20 minutes. In some cases, an infusion of hypertonic mannitol (1.4M, intracarotid bolus – 2ml per 200-250g of animal; Sigma-aldrich, MO) was infused 5 min prior to the fluorescent dextran injections5. Rats were then rapidly decapitated and the brains harvested and post-fixed 48h in 4% phosphate-buffered paraformaldehyde, followed by cryoprotection in 0.01mol/L phosphate-buffered saline containing 30% sucrose for 3 days at 4°C. 40 μm sections containing the hypothalamus and the brainstem were collected. Sections were counterstained with Toto (1:50000) to delineate the nuclei to be studied, and confocal imaging was directly acquired (see below). In some cases, tissue immunohistochemistry was performed for cell type colocalization tests.

Immunohistochemistry

Rats were deeply anesthetized with sodium pentobarbital (100mg/kg, i.p.) followed by transcardial perfusion with 0.01 mol/L phosphate-buffered saline (PBS, 150 ml) and 4% paraformaldehyde (4% PFD - 350 ml) respectively. The dissected brain was post fixed overnight in 4% PFD followed by cryoprotection in PBS containing 30% sucrose for 3 days at 4°C. For protein constituents of BBB analysis, 25μm sections containing hypothalamus and brainstem were collected, pre-incubated in 10% horse serum for 1h and incubated with the following primary antibodies 1) anti-mouse endothelial barrier antigen (EBA, 1:2000; Covance, CA); 2) anti-mouse transferrin receptor (TfR, 1:1000; Invitrogen, CA) for 24h. Immunoreactivity was revealed by 4h incubation in the presence of fluorescently labeled secondary antibody (anti-mouse Cy3-labeled antibody, 1:250, Jackson Immunoresearch; PA). For cell-type identification or microglial marker, sections were incubated with primary antibodies against neuronal cells (anti-mouse NeuN, 1:1000; Millipore, CA); astrocytes (anti-mouse GFAP, 1:1000; Chemicon, CA or anti-mouse S100β, 1:100; Sigma-aldrich, MO) or microglia cells (anti-mouse
CD11B, 1:100; Millipore, CA or anti-mouse ionized calcium-binding adaptor molecule 1 (IBA1, 1:1000; Wako, VA) followed by incubation in secondary antibodies as described (anti-mouse Cy3, 1:250; or anti-mouse Cy5, 1:50; both Jackson Immunoresearch, PA. Every step was preceded by rinses in PBS for 3 times of 5 min. All antibodies were diluted with PBS containing 0.1% Triton X-100 and 0.4% NaN₃. Following the protein constituent EBA immunostaining reaction, the vasculature was counterstained with Isolectin GS-IB4 Alexa Fluor-488 conjugate (1:100; Invitrogen, CA) diluted in 0.01M PBS/0.1% Triton X-100 containing 1mM CaCl₂ and 10mM Hepes (pH 7.4) applied to the sections overnight and washed subsequently. The sections were finally mounted in Vectashield (Vector laboratories, CA) and confocal imaging was acquired.

Confocal imaging acquisition

Acquisition of sections stained with fluorescent dextrans, counterstaining and/or immunofluorescence from the PVN, RVLM and the NTS, were examined with a Zeiss LSM510 confocal scanning microscope (Carl Zeiss, GER) as previously described. An Argon-krypton laser was used to excite FITC10 at 488nm. Helium-neon lasers were used to excite RHO70 and Cy3-labeled secondary antibody at 543 as well as the readily fluorescent Toto counterstaining and Cy5-labeled secondary antibody at 633nm. Images from 12 consecutive optical focal planes (2µm interval) were taken, each optical section was four three times, and a projection image of the sections was generated. To minimize crossover artifacts among the channels, each channel was acquired sequentially and narrow bandwidth emission filters for the different channels were selected. Images from WKY and SHR groups were digitized with identical acquisitions settings for further comparison.

Method for digital quantification of blood brain barrier permeability and protein constituents in brain slices

The permeability status of the BBB was assessed by a modification of a previously described method, based on the detection of fluorescent-dye staining within brain microvasculature or the brain parenchyma. Briefly, two fluorescent-dyes of different fluorophores and molecular sizes were used, a large (RHO70, 70 kDa) and a small one (FITC10, 10 kDa). Even in the presence of an altered BBB permeability, the large RHO70 dye is expected to be contained within the microvasculature, whereas the smaller FITC10 dye is expected to partially leak into the brain parenchyma. This is shown in the example of (Fig.1D) (from an SHR rat), in which RHO70 staining was only observed within microvessels, whereas FITC10 was observed both within microvessels (colocalizing with RHO70, shown as yellow staining in Fig.1D, and white in Fig.1E), but also as either large (solid arrows) or small, punctate accumulations within the brain parenchyma (empty arrows). To detect and quantify the amount of leaked, extravasated FITC10, we used a digital subtraction approach, in which we subtracted from the total FITC10 staining (intravascular + extravascular, Fig.1G), the FITC10 staining located only intravascularly (Fig.1F). This subtraction provided us with the FITC10 staining located only within the brain parenchyma (Fig.1H). Briefly, we started with an image containing all the FITC10 staining (Fig.1G). Secondly, to detect only the FITC contained intravascularly, we used Image J software algorithms (NIH) that detected and isolated
the colocalized RHO70-FITC10 signal (shown in the binary image in Fig.1F). This image then represents the FITC10 located intravascularly. Finally, to isolate the FITC10 that was located only extravascularly, Fig.1F was digitally subtracted from Fig.1G, resulting in a binary image containing only the “leaked” FITC10 signal (Fig.1H). This approach was tested in different sections from different animals, giving consistent and successful results. To quantify the degree of leaked FITC in the subtracted image, the proportion of positive pixels (i.e. representing FITC staining) in the binary image was calculated, and compared among experimental groups.

To compare differences in BBB protein constituents or immunoreactivity between WKY and SHR rats, we performed a densitometry analysis, based on a threshold paradigm as previously described\(^1\). Briefly, an averaged background staining was obtained from each image, and all signal containing an intensity of 2.5 times above the calculated background fluorescence was detected and used for quantification. No differences in mean background levels were observed among the different animals and treatment used. Regions of interest were traced within the different brain nuclei tested, and the density of EBA/ Tfr / CD11b signals within each nucleus was calculated. In all hypothalamic and brainstem sections analyzed, quantifications were performed from both the right and left nuclei. Since no apparent differences were observed between them, data from both hemi-brains were combined, and an average value for each section was used for analysis. Results were expressed as % area within the nucleus occupied by thresholded immunoreactivity.

**Analysis**

Data are presented as mean ± SEM. Unpaired t-tests and one way analysis of variance (ANOVA), followed by Bonferroni posthoc tests, were used as indicated. All analyses were performed using Graphpad prism software. Values of \( P < 0.05 \) were considered statistical significant.
References


7. Mayhan WG. Leukocyte adherence contributes to disruption of the blood-brain barrier during activation of mast cells. *Brain Res*. 2000;869:112-120.
Supplemental Tables, Figures and Figure Legends

Table S1. Antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Abbreviation</th>
<th>Dilution</th>
<th>Source</th>
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<tr>
<td>anti-mouse endothelial barrier antigen</td>
<td>EBA</td>
<td>1:2000</td>
<td>Covance, CA</td>
</tr>
<tr>
<td>anti-mouse transferrin receptor</td>
<td>TfR</td>
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<td>Anti-mouse anti neuronal nuclei</td>
<td>NeuN</td>
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<td>Sigma-aldrich, MO</td>
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<tr>
<td>anti-mouse CD11b</td>
<td>CD11B</td>
<td>1:100</td>
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Table S2. Summary data of extravasated FITC10 (FITC<sub>EV</sub>, % area) in the amygdala, caudate, globus pallidus and somatosensory cortex of WKY and SHR rats (n= 4).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Nuclei</th>
<th>Amygdala</th>
<th>Caudate</th>
<th>Globus pallidus</th>
<th>Somatosensory cortex</th>
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<tr>
<td>WKY</td>
<td></td>
<td>1.13 ± 0.19</td>
<td>1.15 ± 0.21</td>
<td>1.09 ± 0.13</td>
<td>1.62 ± 0.23</td>
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<tr>
<td>SHR</td>
<td></td>
<td>1.36 ± 0.60</td>
<td>1.05 ± 0.27</td>
<td>1.13 ± 0.31</td>
<td>2.23 ± 0.65</td>
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</tbody>
</table>
Figure S1 - **Comparison of extravasated FITC10 in control rats in brain areas lacking or possessing an intact BBB.** Representative image showing intravascularly-delivered FITC10 in the subfornical organ (A) and the area postrema (B), two brain regions located outside the BBB. In C and D, a representative image of FITC10 in the PVN and SON, respectively, two areas located inside the BBB, are also shown. The inset in B shows the squared area at a higher magnification. Scale bar: 25 µm for A, C, D and inset on B. 50 µm for B. 3V and 4V indicate third and fourth ventricle, respectively.
Figure S2 - **A hyperosmotic intravascular stimulus disrupts BBB permeability.** A, Representative photomicrograph showing intravascularly-delivered RHO70 (red) and FITC10 (green) in the PVN of a rat that previously received an intravascular injection of hypertonic mannitol (1.4M). The squared area in A is shown at a higher magnification in B. Note the presence of extravasated FITC10 either in the neuropile (empty arrows) or within perivascular cells, possibly pericytes (solid arrows). Scale bar: 50 µm for A, 25 µm for B.
Figure S3 - **Increased BBB permeability within the PVN, NTS and RVLM nuclei in renovascular hypertension rats.** Photomicrographs showing staining for intravascularly-delivered RHO70 (red) and FITC10 (green) within the PVN, NTS and RVLM of Sham (A1, B1, C1) and RVH (A2, B2, C2) rats, respectively. Examples of isolated extravasated FITC10 for Sham and RVH are shown in A3, A4 for PVN; B3, B4 for NTS and C3, C4 for NTS. A5, B5 and C5 shows mean extravasated FITC10 (FITC10\(_{EV}\)) in Sham and RVH rats within the respective nuclei. *P<0.05 and **P<0.001 vs. Sham. n=3 RVH/Sham. Scale bars: 50µm. 3V: third ventricle.
Figure S4 - **Co-localization of intravascularly-delivered AngII\textsubscript{fluo} with microglia, but not with astrocytes in SHR rats.** A-C, Intravascularly-delivered AngII\textsubscript{fluo} (A, green) and the astrocytic body marker S100\(\beta\) (B, blue) shows no co-localization between both markers within the PVN (C, merged). D-I similarly, no co-localization was found between the leaked intravascularly-delivered AngII\textsubscript{fluo} (D,G, green) with the astrocytic process marker GFAP (E,H, blue) within the NTS (panel F merged) and RVLM (panel I merged). Scale bar: 20 \(\mu\text{m}\).
Figure S5 - Diminished transferrin receptor (TfR) expression in SHR rats. A-F, Photomicrographs showing transferrin receptor (TfR) immunostaining within the PVN, NTS and RVLM of WKY (A-C) and SHR (D-F) rats. G, Mean TfR immunoreactivity density. *P<0.05 vs. respective WKY. Scale bar: 50µm. 3V: third ventricle.
Figure S6 - Endothelial barrier antigen (EBA) and Transferrin receptor (TfR) fluorescence intensity. A and B shows mean of EBA and TfR immunoreactivity intensity in WKY and SHR within the PVN, NTS and RVLM respectively. No differences were observed within the nuclei studied (n= 5 rats each group).