

## **Online Supplement**

### **Aldosterone Acting through the CNS Sensitizes Angiotensin II-Induced Hypertension**

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## **Methods**

### **Measurement of mRNA Expression in the LT and PVN**

Total RNA was isolated from LT and PVN using Trizol method (Invitrogen) and treated with DNase I (Invitrogen). RNA integrity was checked by gel electrophoresis. Total RNA was reverse transcribed using random hexamers following the manufacturer's instructions (Applied Biosystems). Real time PCR was conducted using 200-300 ng of cDNA and 500 nM of each primer in a 20  $\mu$ l reaction with iQ SYBR Green Supermix (Bio-Rad). Amplification cycles were conducted at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and annealing/extension at 60°C for 30 s. Reactions were performed in duplicate and analyzed using a C1000 thermocycler system (Bio-Rad). Samples that did not yield homogenous melt curves were excluded. Changes in mRNA expression levels were normalized to GAPDH levels and calculated using the  $\Delta\Delta C_t$  method. Results are expressed as relative fold change, mean of fold change  $\pm$  SE. Primers were purchased from Integrated DNA Technologies (Coralville, IA). The sequences of the primers are shown in Table S1.

### **SFO cells culture**

Five to six rat pups (18-20 days old) were decapitated, and the head was placed in ice-cold 70% ethanol. The brain was removed immediately and placed in a petri dish with ice-cold cutting solution (220 mM sucrose, 3 mM KCl, 0.2 mM CaCl<sub>2</sub>, 10 mM dextrose, 6 mM MgSO<sub>4</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>). A thick coronal slice was made to include tissue from the rostral level of the optic chiasm caudal to the collicular level. Previous dissection studies carried out in animals that had been systemically injected with Evans blue (1% solution), which labels brain areas outside the blood-brain barrier (i.e., circumventricular organs, such as the SFO), have shown that the SFO can be isolated and removed from these coronal slices with minimal non-SFO tissue attached (non-SFO tissue is mainly from the hippocampal commissure). Isolated SFOs from five to six pups were transferred to a tube containing Earle's balanced salt solution (Sigma) and dispase I (4 U/2 ml; Roche) and incubated for 1 h at 37°C. After three washes in culture medium, a cell suspension was prepared by trituration of the fragments through a fire-polished Pasteur pipette until the tissue fragments were visibly dissociated, and then several drops of the medium containing the cells were plated onto previously precoated (0.1 mg poly-L-lysine/ml; Sigma) round 22-mm coverslips and incubated in a humidified atmosphere (plus 5% CO<sub>2</sub>) at 37°C. The culture medium was Dulbecco's modified Eagle's medium (Sigma) with 10% fetal bovine serum (heat inactivated at 56°C for 30 min; Sigma) and 1% L-glutamine-penicillin-streptomycin solution (Sigma) added. After 2-4 h postplating to allow the cells to adhere to the coverslips, additional medium was added.

### **Intracellular calcium measurement**

Intracellular calcium concentration was measured with Fluo-4AM. Cells were loaded with Fluo-4 by incubating cells grown on 22-mm coverslips in artificial cerebrospinal fluid (aCSF; 126 mM NaCl, 3 mM KCl, 2 mM CaCl<sub>2</sub>, 10 mM dextrose, 1 mM MgSO<sub>4</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>) containing 0.02 mM Fluo-4 for 60 min at 37°C. During the incubation, fluo-4-AM is absorbed by the cell and hydrolyzed only within living cells to impermeant Fluo-4. For cytosolic calcium measurements and pharmacological manipulations, the coverslip was mounted in a bath chamber that was attached to the microscope stage and artificial CSF was used as the recording

solution. Fluorescence intensity was measured using a video microscopic digital image analysis system (FluoView 500 Confocal Laser Scanning Microscope; Olympus) with a 488 nm Argon laser as an excitation source. An increase in cytosolic calcium concentration produces an increase in fluorescence intensity with only a little shift in wavelength. At the end of the experiment data was analyzed and the fluorescence intensity converted to numerical values. A response to stimulation was discerned as a peak increase in fluorescence intensity and represented as a percentage change from baseline recording. Cell viability was checked at the end of the experiment by applying artificial CSF containing 50 mM KCl (substituting an equimolar amount of NaCl). All procedures were performed at room temperature ( $24 \pm 2^{\circ}\text{C}$ ) in a dark room. The manipulation of removing and applying bath solutions can induce a small change in fluorescence intensity ( $2.99 \pm 0.67\%$ ). Therefore, only cells that presented a change in fluorescence intensity greater than 8.7% (average plus two standard deviations) were considered as being responsive to the stimulus applied.

### Immunohistochemistry

The day after the conclusion of the treatments, the rats were anesthetized and perfused with 0.1 M PBS, followed by 4% paraformaldehyde (PF). Free-floating sections (40  $\mu\text{m}$ ) were incubated in 5% normal donkey serum for 1 h, followed by a primary antibody, rabbit polyclonal anti-Fra (K-25, 1:1000, Santa Cruz) in 5% donkey normal serum with 0.2% Triton X-100 for 72 h at  $4^{\circ}\text{C}$ . After being thoroughly washed with PBS, sections were incubated with Cy<sup>TM</sup>2-conjugated AffiniPure donkey anti-rabbit IgG (1:100, Jackson) in PBS for 2 h at room temperature. Fluorescence was then identified using confocal microscopy. In the PVN, level 1 (-1.60 mm) is the most rostral and included the dorsal parvocellular, medial parvocellular, and ventrally located posterior magnocellular subnuclei. Level 2 (-1.88 mm) contains a prominent posterior magnocellular region and both dorsal and ventrolateral parvocellular divisions. Level 3 (-2.12 mm) is the most caudal and consisted of the medial and lateral parvocellular divisions. The number of retrogradely labeled cells from the spinal cord and RVLM is significantly greater at levels 2 and 3 vs level 1 (Stocker et al, *Am J Physiol Regul Integr Comp Physiol* 287:R1172-R1183, 2004). We used levels 2 and 3 for summarizing the Fra results.

**Table S1: Primer Sequences for Real Time PCR**

Gene	Forward primer	Reverse primer	Product size(bp)	Accession number
GAPDH	TGACTCTACCCACGGCAAGTTCAA	ACGACATACTCAGCACCAGCATCA	141	XM_001062726.2
Renin	CTGCCACCTTGTTGTGTGAG	ACCTGGCTACAGTTCACAACG	154	NM_012642.4
AGT	TCCCTCGCTCTCTGGACTTA	AAGTGAACGTAGGTGTTGAAA	209	NM_134432.2
AT1R	CTCAAGCCTGTCTACGAAAATGAG	GTGAATGGTCCTTTGGTCGT	188	NM_030985.4
AT2R	ACTTTTGAACATGGTGCTTTG	TTTCTATGCCAGTGTGCAG	160	NM_030985.4
ACE1	GTGTTGTGGAACGAATACGC	CCTTCTTTATGATCCGCTTGA	187	AF539425.1
ACE2	TAAAGCCACCTTACGAGCCTC	GCCAATGTCCATGGAGTCAT	170	GQ262788.1
MR	GCCCGGCAAATCTCAACAACCTCAA	TTAGGGAAAGGAACGTCGTGAGCA	235	M36074
AS	TATAGAAGCCAGCAACTTTGCAC	AGTCAAGCTTCTGGGTAAGAACAG	148	NM_012538

AGT, angiotensinogen; MR, mineralocorticoid receptor; AT-R, angiotensin receptor; ACE, angiotensin converting enzyme; AS, aldosterone synthase