Online Supplement:
Parallel Changes in Neuronal AT1R and GRK5 Expression Following Exercise Training in Heart Failure

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Supplemental Methods

Induction of Heart Failure. Briefly, all rats were anesthetized with isoflourane (0.5-2% in oxygen) intubated, and mechanically ventilated. Under sterile conditions, a left thoracotomy was performed through the fifth intercostal space. Subsequently, the pericardium was opened and the heart was exteriorized. The left anterior descending coronary artery was ligated with a 6–0 prolene suture that was passed through the superficial layers of myocardium, between the pulmonary artery outflow tract and left atrium. Following ligation, the heart was placed in its original position, and the thorax was closed. The air within the thorax was evacuated, allowing the rats to resume spontaneous respiration and recover from anesthesia. Analgesia (Buprenorphine, Reckitte Benckiser, Hull, UK; 0.1 mg/kg, sc) was administered post-surgically. Sham-operated rats were prepared in the same manner but did not undergo coronary artery ligation. Left ventricular dysfunction was assessed using hemodynamic and anatomic criteria. Echocardiograms were performed (Vevo 770; Visualsonics, Inc.) before, during, and after the six week ExT period. Rats with ejection fraction as determined by echocardiogram of less than 50%, were considered to be in HF.

Exercise Training Protocol. Initially, a low speed (10 m/min) and grade (0%) and short duration (10 min/day; 5 days/week) was used to familiarize the rats with running on the treadmill. The speed, duration, and grade were gradually increased to 20-25 m/min, 60 min/day, and 5-10%, respectively, to ensure that a significant endurance effect was produced. This level of exercise is considered moderate for the sham rats [1]. Only rats that ran steadily with little or no prompting were used in the study. To ensure a similar level of ExT between groups and to document a training effect, in some animals, citrate synthase activity assays on the soleus muscle were performed following the protocol of Srere [2]. In a subgroup of animals, an ExT effect was determined by the duration of time the animal could run continuously without lagging or stopping. This testing was performed both pre- and post-ExT or Sed periods.

Metabolic Cage Assessment and Measurement of Urinary Norepinephrine Excretion. Rats were placed in a metabolic cage to measure water and food intake, urine and fecal excretion, and body weight for 72-96 hour intervals pre- and post-ExT. The urine was collected under mineral oil and frozen (–80°C) until it was used for the measurement of norepinephrine concentration. This measurement was done using a Norepinephrine Enzyme Immunoassay kit (Labor Diagnostika Nord GmbH & Co KG, Montreal, Quebec) according to the manufacturer's instructions.

Micropunch of the PVN and RVLM and isolation of protein for Western blot measurements. After euthanization, brains were removed and quickly frozen on dry ice. Coronal sections were cut through the hypothalamus and medulla at the level of the PVN and RVLM respectively using a cryostat and, following the Palkovits technique [3], the nuclei were bilaterally punched using a diethylpyrocarbonate (DEPC)-treated blunt 18-gauge needle attached to a syringe. Punches were lysed in 200 μL of RIPA buffer with fresh protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and total protein concentration was measured using a BCA Assay kit [4].

Cell Culture and Maintenance. For overexpression experiments, a GRK5 in pcDNA3 plasmid (a generous gift from Dr. Jeffrey Benovic) was transfected into confluent CATH.a cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) reagent according to manufacturer's instructions. GRK5 knockdown using siRNA (Santa Cruz) transfection were also performed using Lipofectamine 2000 according to manufacturer's instructions.

Western blot measurement of proteins. Samples were adjusted to contain the same concentration of total protein, and then equal volumes of 2X 4% SDS sample buffer were added. The samples were boiled for 3 min and then loaded onto a 7.5% SDS-PAGE gel (40 μg/20 μl per well). Gels were subjected to electrophoresis at 115 V/gel for 60 min. The fractionated proteins on the gel were electrophoretically transferred to a PVDF membrane (Millipore, Billerica, MA) at 50 V for 90 min. The membrane was probed with any of the primary antibodies overnight: rabbit anti- phosphothreonine (1:500, Abcam, Cambridge, MA), goat or rabbit anti-AT1R, rabbit anti-GRK5, rabbit anti-GRK2, mouse anti-p65 NF-κB, and/or mouse anti-GAPDH (1:500-1:1,000, Santa Cruz). Although the commercial availability of the antibodies should imply specificity, we performed blocking peptide experiments for the GRK5 antibody in both PVN and CATH.a lysates (Supplemental Figure 1). Following thorough washes with PBST, the samples were then probed with the appropriate secondary antibodies (Li-Cor Biosciences, Lincoln, NE). Blots were developed using a Li-Cor Odyssey scanner and quantitative analysis of band densitometry was performed using the Li-Cor Odyssey software. The relative abundance of proteins of interest was calculated as the ratio of intensity of the band relative to the intensity of GAPDH. Graphs summarizing individual experiments are shown as a fold change compared to the Sham Sed animals or fold change compared to non-stimulated cells. Preparation of samples and co-immunoprecipitation experiments can be found in the online supplement.

Co-immunoprecipitation. In order to determine the interaction between AT1R and GRK5 co-immunoprecipitation (co-IP) experiments were carried out. In co-IP experiments, equivalent amounts of PVN total protein lysate were tumbled with Roche Protein G beads (Roche, Indianapolis, IN) and pre-cleared for three hours. After centrifugation (20 seconds, 12,000 x g), the pre-cleared lysate was allowed to tumble with appropriate antibody (either goat anti-AT1R or rabbit anti-GRK5, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr prior to the addition of Protein G beads. The conjugated beads and lysate were tumbled overnight at 4°C. The beads were washed with two separate washing buffers (Buffer 1: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Tween-20, 0.05% Na Deoxycholate; Buffer 2: 50 mM Tris-HCl pH 7.5, 75 mM NaCl, 0.1% Tween-20) with protease inhibitor cocktail added on the day of experimentation. Protein was eluted from the beads using 50μL of 2X 4% SDS sample buffer with β-mercaptoethanol (2.5% final concentration) added fresh and heated to 100°C prior to western blotting.

1. Musch TI, Terrell JA. Skeletal Muscle Blood Flow Abnormalities in Rats with a Chronic Myocardial Infarction: Rest and Exercise. *Am J Physiol.* 1992;262:H411-H419.
2. Srere PA. Citrate Synthase. *Methods Enzymol*. 1969;13:3-11.
3. Palkovits M, Brownstein M. Brain Microdissection Techniques. In: Cuello AE, ed. *Brain Microdissection Techniques*. Chichester: John Wiley & Sons; 1983.
4. Kleiber AC, Zheng H, Schultz HD, Peuler JD, Patel KP. Exercise Training Normalizes Enhanced Glutamate-Mediated Sympathetic Activation from the Pvn in Heart Failure. *Am J Physiol Regul Integr Comp Physiol*. 2008;294:R1863-1872.

Table S1. Echocardiographic, baseline body weight, and heart weight data.

parameter	**sham sed**	**sham ExT**	**CHF Sed**	**CHF ExT**
	(n=5)	(n=5)	(n=12)	(n=7)
LVDd (mm)	7.8 ± 1.2	7.2 ± 0.8	8.9 ± 1.2 *	9.3 ± 1.3 *
LVDs (mm)	4.7 ± 0.9	4.04 ± 0.6	6.9 ± 0.9 *	7.1 ± 1.1 *
LVd Vol (µL)	327.9 ± 109	277.8 ± 59.4	438.1 ± 149.4	491.3 ± 154.6 *
LVs Vol (µL)	106.3 ± 48.9	76.6 ±26.5	243.8 ± 80.4 *	269.7 ± 97.5 *
EF%	68.4 ± 6.4	72.9 ± 4.9	44.5 ± 3.5 *	45.1 ± 5.9 *
FS%	39.9 ± 5.3	43.5 ± 4.1	23.2± 2.2	23.6 ± 3.6
BW (g)	443 ±10	388 ± 17 †	432 ± 11	386 ± 11†
HW/BW (mg/g)	3.3 ± 0.2	4.3 ± 0.5	4.4 ± 0.1 *	4.4 ± 0.3‡

Data are means±SE. * $p<0.05$ compared to sham, † $p<0.05$ compared to sed, ‡ $p=0.0514$ compared to sham. LVDd, Left ventricular end diastolic diameter; LVDs, left ventricular end systolic diameter; LVd Vol, left ventricular end diastolic volume; LVs Vol, left ventricular end systolic volume; EF%, Ejection Fraction; FS%, fractional shortening; HW/BW, heart weight/body weight ratio.

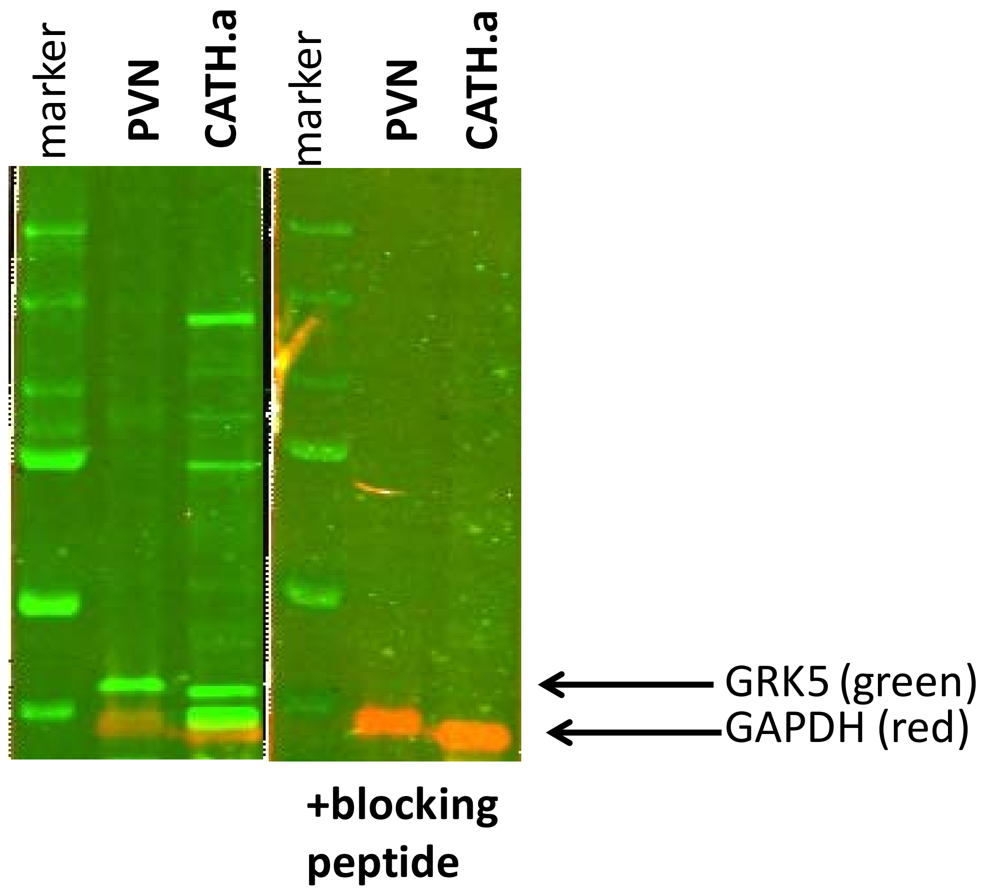
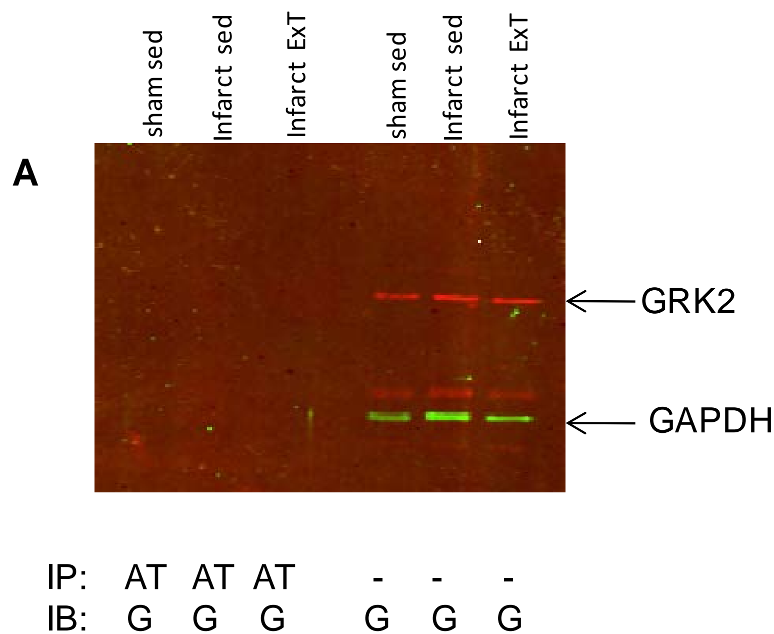


Figure S1. GRK5 antibody is specific in both PVN micropunch lysates and CATH.a neurons.



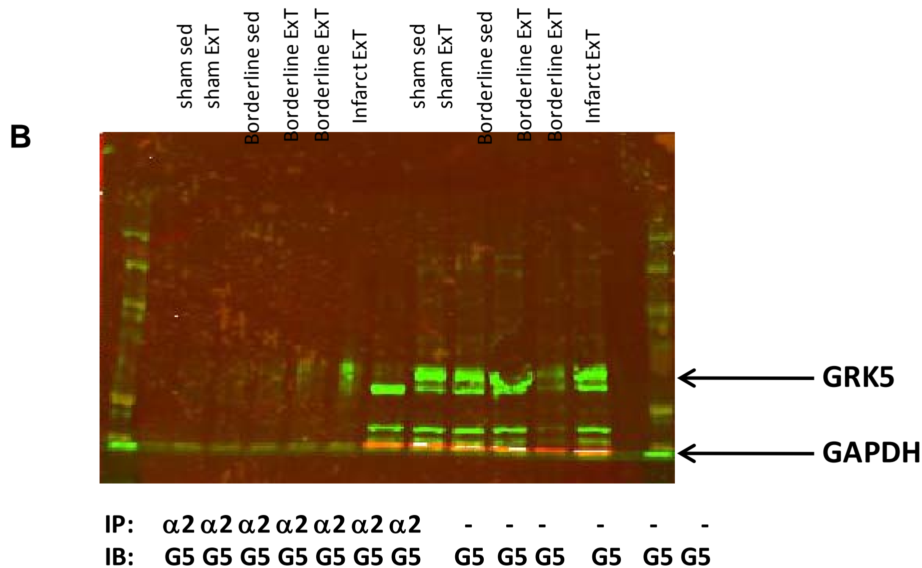


Figure S2. GRK2 and AT1R α2c-AR and GRK5 do not physically associate. A. In co-immunoprecipitation experiments in PVN micropunches, lysates were pulled down with an anti-AT1R antibody (AT), and IP samples were run alongside total protein lysates. Immunoblotting with an anti-GRK2 antibody (G) indicates that AT1R and GRK2 do not physically associate, as no positive bands were detected in any of the IP lanes. Both GRK2 (shown in red) and GAPDH (shown in green) were detected in all lanes containing total lysate. B.Lysates were pulled down with an anti-α2c-AR antibody and IP samples were run alongside total protein lysates. α2c-AR and GRK5 do not physically associate, but both GRK5 (shown in green) and GAPDH (shown in red) are detected in total lysate.

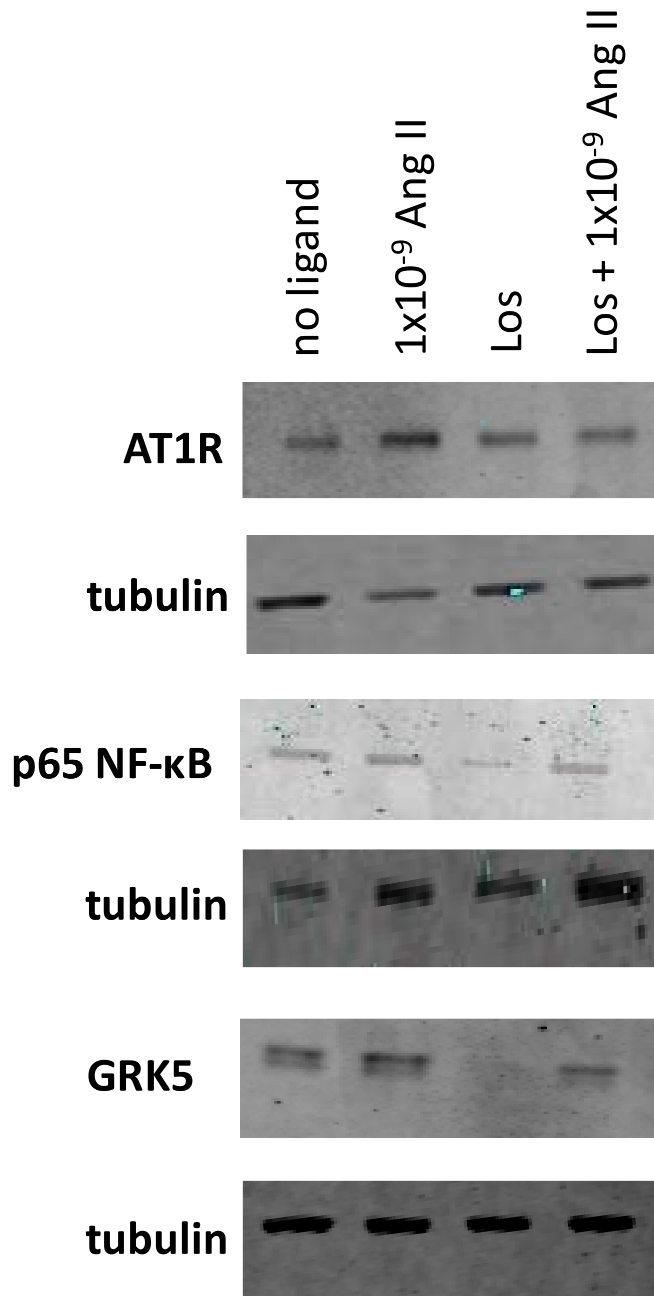


Figure S3. Lower dose of Ang II elicits same changes in AT1R, p65 NF-κB and GRK5. Western blots of CATH.a neuron total lysates stimulated with 1 nM Ang II, 100 nM Los, or 1 nM Ang II plus 100 nM Los. Stimulation with 100-fold lower dose of Ang II still increases AT1R, p65 NF-κB and GRK5 proteins.