ONLINE SUPPLEMENT:

Glutamatergic Receptor Activation in the Rostral Ventrolateral Medulla Mediates the Sympathoexcitatory Response to Hyperinsulinemia

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Material and Methods for Western Blot Analysis of Insulin Receptor β

Frozen RVLM or hypothalamus sections were disrupted in 150 μl of homogenization buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaCl, 400 mM KCl, 25 mM β-glycerophosphate, 50 mM NaF, 5 mM benzamidine, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM sodium orthovanadate, 5 mM N-ethylmaleimide, 1 mM PMSF) supplemented with protease inhibitor cocktail (P8340; Sigma, St Louis, MO)] using a Pyrex Potter-Elvehjem tissue grinder. To remove any insoluble particulate the tissue homogenates were centrifuged (10,000 x *g*, 10 min, 4 °C) and the supernatant transferred to a new microcentrifuge tube. Protein concentration of each sample was determined using the Bio-Rad DC protein Assay (Hercules, CA) according to the manufacturer's directions. Five micrograms of each sample was precipitated using the methanol:chloroform procedure and then resuspended in 20 μl of 1X sample buffer. Samples were prepared for electrophoresis by heating for 5 min at 100 °C. Samples were separated by SDS-PAGE (7.5% gel) and then transferred to nitrocellulose membrane (0.2 μm) (Bio-Rad, Hercules, CA). The membrane was incubated in blocking buffer (5% nonfat dry milk in TBS plus 0.1% Tween-20 [TBS-T]) for 1 hr at room temperature and then incubated in blocking buffer overnight at 4°C with a rabbit polyclonal insulin receptor β (IR-β)antibody (1:1000, C-19; sc-711; Santa Cruz Biotechnology, Inc. Santa Cruz, CA.). After the overnight incubation, the membrane was washed (4x, 5min) in TBS-T, incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000, 1 hr) at room temperature, incubated in ECL plus for 5 min (GE Healthcare, Piscataway, NJ) and exposed to X-ray film. To determine specificity of the IR-β antibody, the membrane was stripped and reprobed with the IR-β antibody pre-absorbed with five-fold excess of a blocking peptide (sc-711P; Santa Cruz Biotechnology, Inc. Santa Cruz, CA.). The membrane was also stripped and reprobed with a mouse monoclonal γ-tubulin antibody (T6557; Sigma-Aldrich, St. Louis, MO). The IR-β band intensity for each sample was quantified using NIH ImageJ (http://rsb.info.nih.gov/nih-image/) and normalized to the respective γ-tubulin band intensity.

Table S1. Characteristics for LF, OR, and OP rats

	Group		
Characteristic	LF	OR	OP
Initial Body Weight, g	221±7	233±2	245±4*
Final Body Weight, g	612±16	596±8	793±13*
Fat Pads			
Epididymal, g	14±1	16±1	28±1*
Retroperitoneal, g	20±3	21±1	38±2*
Total, g	34±4	36±3	67±3*
Adiposity Index, %	5.5±0.6	6.1±0.4	8.4±0.4*

Values are mean ± SEM. *Significant difference versus LF or OR rats (P<0.05).

Table S2. Characteristics of Lean and Obese Zucker Rats

Characteristic	Lean (n=8)	Obese (n=8)
Age (weeks)	15.3±0.5	15.8±0.4
Body Weight (g)	376±9	564±21*
Insulin (ng/mL)	1.1±0.2	10.5±3.5*

Values are mean ± SEM. *Significant difference versus lean ($P<0.05$).

Plasma insulin levels were analyzed from samples of lean and obese Zucker rats generously provided by Dr. David Stepp (Medical College of Georgia). Half the rats were fasted overnight. Then, rats were anesthetized with isoflurane, decapitated, and trunk blood collected. Insulin levels were determined by an ELISA using a commercially available kit (Millipore).

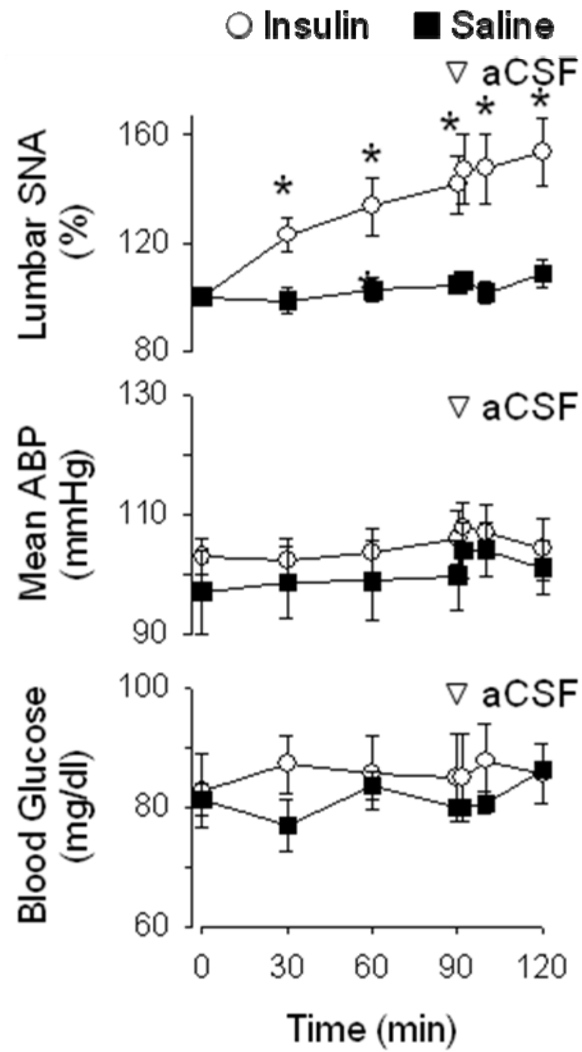

Figure S1. Summary figures of lumbar, mean ABP, and blood glucose during 120-minute hyperinsulinemic-euglycemic clamp or saline infusion. ○ Insulin + aCSF (n=7) and ■ saline + aCSF (n=3). *Significant difference vs saline ($P<0.05$)

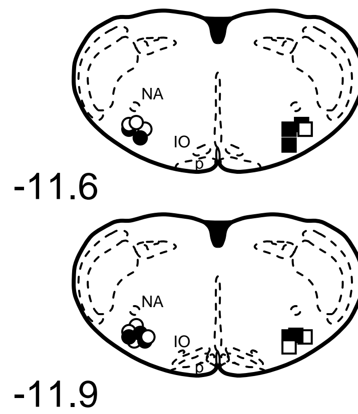

Figure S2. Schematic drawings of RVLM injection sites (● Insulin + KYN, ○ insulin + aCSF, ■ saline + KYN, and □ saline + aCSF). Microinjections of AP5, NBQX, losartan and SHU9119 were similar in location (data not shown). Sections represent -11.6 mm (top) and -11.9 mm (bottom) in reference to bregma. IO indicates inferior olive; p, pyramidal tracts; NA, nucleus ambiguous; ST, spinal trigeminal nucleus