Online Supplement

Selective Deletion of Renin-b in the Brain Alters Drinking and Metabolism

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Running title: Altered Metabolism in Renin-b Deficient Mice

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Supplemental Methods

Drinking Studies. Mice (15-19 weeks of age) were individually housed in metabolism cages after body weight was recorded. Mice were acclimatized for 2 days. Food intake, fluid intake, and urine output was measured for 2 days at baseline and again for 2 days after DOCA-salt. The DOCA-salt model of hypertension was performed as we previously described.[1] Note, this model of DOCA-salt hypertension does not involve a uninephrectomy. Fluid was a two bottle choice paradigm consisting of water and 0.15M saline as we reported previously.[2,3] In order to further stimulate the brain RAS, the mice were deprived of water for 18 hours and then cumulative water intake was measured for 2 hours under baseline conditions. After a recovery period, DOCA-salt was administered and the experiment was repeated 21 days later.

Blood Chemistry. Blood chemistry was measured by iSTAT using CHEM8+ and CG4+ cartridges but there were no differences in blood pH, PCO_2, PO_2, HCO_3, lactate, sodium, potassium, chloride, calcium, or glucose in control or Ren-b^{Null} mice either under baseline or after DOCA-salt. There was also no difference in blood urea nitrogen, creatinine, or hematocrit.

Urinary Corticosterone and Aldosterone. Mice were housed in metabolic cages for 2 days for urine collection. Normal chow and water were provided *ad libitum* during the entire experiment. Animals were acclimatized to the new environment during the first day in the metabolic cages and urine was collected and measured during the subsequent 24 hours. Urinary corticosterone (Catalog 501320) and aldosterone (Catalog 501090) concentrations were measured in duplicate using selective commercially available ELISA kits (Cayman Chemical; Ann Arbor, MI) following the manufacturer's instructions. Urinary corticosterone or aldosterone excretion rate was expressed as nanograms per day by multiplying the concentration X total urine volume.

Metabolic Measures and Calculations. Body composition was evaluated using nuclear magnetic resonance (NMR; Bruker LF90II). Food intake and fecal output were measured daily in metabolic cages. Caloric densities of food and fecal samples were determined using a semi-micro bomb calorimeter (Parr) as previously and reviewed in detail.[4] Physical activity was assessed using radiotelemetric probes (DSI). Resting metabolic rate (RMR) was determined by push-pull respirometry as previously described.[1,3]

To determine resting metabolic rate, Mice were placed into a thermally-controlled 2L chamber with a plastic floor, maintained in the middle of the thermoneutral temperature zone for C57 mice (30°C). Air was passed through the chamber at a fixed rate (~300 mL/min corrected to STP), and the chamber's effluent airstream was assessed for temperature, relative humidity, pressure, and mass flow before being subsampled (~150 mL/min STP), and passed through a desiccant column ($CaSO_4$, Drierite) and into a continuously-sampling oxygen (O_2) / carbon dioxide (CO_2) analyzer system (AEI). Gas analyzers were calibrated daily using soda lime and commercially-produced standard gas mixtures as previously described.[5] STP-corrected air flow, and changes in effluent

air O_2 / CO_2 composition between bouts of sleep and the pre-/post-test baseline were then used to estimate RMR using the equation derived from Lusk.[6]

Calories consumed were calculated by:

$$Calories\ consumed = (Mass\ of\ food\ consumed) \times (Caloric\ density\ of\ food)$$

Calories lost to feces were calculated as:

$$Calories\ lost\ to\ feces = (Mass\ of\ feces\ produced) \times (Caloric\ density\ of\ feces)$$

Caloric absorption was calculated by:

$$Caloric\ absorption = (Calories\ consumed) - (Calories\ lost\ to\ feces)$$

Metabolic rate is calculated as kilocalories per hour. The STP-corrected rate of oxygen consumption (VO_2) is calculated as milliliters of O_2 per minute.

$$Metabolic\ rate = VO_2 \times (1.232 \times RER + 3.815)$$

The respiratory exchange ratio (RER) is a unit-less value that generally reflects fuel utilization, and is calculated as ratio of rates of CO_2 production (VCO_2) and VO_2:

$$RER = \frac{VCO_2}{VO_2}$$

NMR: Time-domain nuclear magnetic resonance (NMR)-based quantification was used to assess body composition (relative proportions of fat mass, lean mass, and free fluid mass) with a Bruker LF-50 Body Composition Analyzer.[4] This technology is essentially analogous to a magnetic resonance imager (MRI) in that it uses the tissue-type specific relaxation time of protons throughout a sample to assess the relative proportions of each tissue type in a sample, but yields quantifications of 3-dimensional space rather than 2-dimensional images of the subject. NMR-based methods are generally considered to exhibit improved accuracy and precision compared to X-ray based DEXA methodologies. An anesthetized mouse is placed into a restraint tube,; the tube is placed into NMR device for ~1 minute scan, then the mouse is returned to home cage.

Western Blot: Interscapular brown adipose tissue was homogenized in lysis buffer containing 50 mmol/l Tris Cl buffer, 0.1 mmol/l EDTA (pH 7.5), 1% wt/vol NA deoxycholic acid, 1% vol/vol NP-40 and 0.1% vol/vol SDS, with cOmplete protease inhibitors and PhosSTOP phosphatase inhibitors (Roche). Homogenates were centrifuged at 4 °C, and supernatant total protein was quantified using Lowry assay. Equal amounts of proteins (30 µg) were separated by electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane (GE healthcare). The membrane was immunoblotted with primary antibody followed by horseradish peroxidase-conjugated secondary antibodies, and protein bands were detected using ECL (Amersham Biosciences).

RNA: Total RNA was extracted using TRIzol (Invitrogen) and isolated using RNA Purelink Minikit (Invitrogen) as per the manufacturer protocol. RNA was then treated with DNase I (Invitrogen), and cDNA generated by reverse transcriptase using SuperScript III using oligo-dT as primer (Invitrogen). β-actin was used as internal control.

UCP1 Protein and mRNA. UCP1 protein levels in brown adipose tissue were evaluated by Western blot analysis using the following antibodies: UCP1, Santa Cruz Biotechnology, UCP1 (C-17), sc-6528; Secondary for UCP1, Santa Cruz Biotechnology, donkey anti-goat IgG-HRP, sc-2020; GAPDH, Santa Cruz Biotechnology, GAPDH (6C5), sc-32233; Secondary for GAPDH, GeneTex, EasyBlot anti-mouse IgG (HRP), GTX221667-01. UCP1 mRNA expression was measured by real-time RT-PCR. Gene expression was determined using SYBR-green assays and primer sets from Integrated DNA Technologies: UCP1, forward 5'-GTGAAGGTCAGAATGCAAGC-3' and reverse 5'-AGGGCCCCCTTCATGAGGTC-3'; β-actin, forward 5'-CATCCTCTTCCTCCCTGGAGAAGA-3' and reverse 5'-ACAGGATTCCATACCCAAGAAGGAAGG-3'.

Brown Adipose Tissue Sympathetic Nerve Activity. Sympathetic nerve recordings were performed as previously described.[7,8] Mice were anesthetized using ketamine/xylazine and instrumented with a colonic temperature probe. The left carotid artery was cannulated concurrently to measure arterial pressure and jugular vein was cannulated to maintain anesthesia with α-chlorolose. The brown adipose pad was exposed and sympathetic nerve was isolated and suspended on a 36-gauge platinum-iridium electrode and secured in place with silicone gel (World Precision Instruments). Electrodes were attached to a high-impedance probe (HIP-511, Grass Instruments), and the nerve signal was amplified (10^5) with a Grass P5 AC preamplifier, filtered at a 100 and 1000 Hz cutoff, and routed to a resetting voltage integrator (model B600c, University of Iowa Bioengineering). The remaining activity after death was considered noise and subtracted from the measurements to determine real SNA. Data were recorded and analyzed using a PowerLab unit and associated Chart software (ADInstruments) on a Macintosh computer.

Glucose and Insulin Tolerance Tests. Glucose tolerance test (GTT) and Insulin tolerance test (ITT) were performed using a protocol we previously described [9]. Mice were individually housed and fasted for 5 hrs. Water was provided *ad libitum*. Body weight and basal glucose levels were obtained before glucose (1 or 2 g/kg BW; D-glucose, anhydrous; Sigma Aldrich) or insulin (0.5 or 1 U/kg BW; Humulin R, regular insulin human injection; Lilly USA) intraperitoneal administration. Blood glucose was measured 15, 30, 60, 90, 120, 150, and 180 min after injection of glucose or insulin. Blood samples were obtained from tail veins in conscious mice and blood glucose levels were determined using a glucometer (One Touch Ultra 1, LifeScan, Inc.).

Glucose Uptake Analysis. [^{18}F]Fluorodeoxyglucose (FDG), a positron-emitting labeled glucose analogue, is utilized clinically to map glucose metabolism in the brain, heart, and tumors and is known to accumulate in brown adipose tissue (BAT) in both humans and mice [10-12]. The degree of FDG accumulation in the BAT is dependent not only on the metabolic activity of the tissue but also the conditions under which the animal is being imaged [13]. Therefore, all mice were carefully and consistently handled prior to tracer injection, during uptake and imaging. Imaging was performed according to methods we previously reported.[14] Specifically, mice were fasted (with access to water)

for a minimum of 12 hours prior to imaging. Animals were weighed and measurements of the blood glucose levels were made using a clinical glucometer from a 0.3 microliter blood sample acquired from the tail (Freestyle glucometer, Abbott Labs, Abbott Park, Illinois). Prior to injection of FDG, the mice were warmed. Mice were injected awake with FDG (7.9 MBq ± 0.32) via tail vein. The animals were returned to their cage for the 60 minute uptake time period. At the end of the uptake period, the mice were anesthetized with Isoflurane (4% induction and 2.5% maintenance) and placed prone on the warmed (36.1^{o} C) bed (m2m Imaging, Cleveland, OH) of the Inveon Docked PET-SPECT-CT system (Siemens Medical, USA) for static PET imaging (with CT for attenuation correction and anatomical localization). Images were reconstructed using a three-dimensional OP-MAP algorithm (Siemens Medical Solutions USA, Inc., Malvern, PA, USA). All image analyses were performed using the VIEW tool of the PMOD Biomedical Quantification Package (version 3.3, PMOD Technologies, Ltd., Zurich, Switzerland). Volumes-of-interest (VOIs) were manually drawn encompassing the interscapular brown adipose tissue (BAT) space on each individual mouse's whole-body CT image that was co-registered with the FDG image. The uptake of FDG was measured in standardized uptake value (SUV) units. This metric corrects for the dose administered and the size (weight) of the animal. However, in a number of animals, there was obvious retention of tracer in the tail surrounding the site of injection. Because this portion of the dose would not be available to the systemic circulation, a second VOI over the retained tracer was created and the residual activity was quantitated in order to create a systemically-available dose for the calculation of a corrected BAT SUV (SUVcorr).

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Table S1: Fluid Phenotypes in Ren-b Mice at Baseline and DOCA-salt

Treatment	Strain	Age (wk)	BW (g)	Fluid (mL/d)	Fluid (mL/g/d)	Na (mEq/d)	0.15M Na Pref (%)	Urine (mL/d)	Urine (mL/g/d)	UNaV (mEq/d)	UKV (mEq/d)	Urinary Corticosterone (ng/day)	Urinary Aldosterone (ng/day)
Baseline	Control (n=8)	16.8±0.5	27.9±0.9	2.8±0.4	0.1±0.01	0.6±0.1	42.8±9.5	1.0±0.2	0.04±0.01	0.4±0.1	0.4±0.05	60.0±8.0	24.2±3.1
	Ren-b^{Null} (n=6)	15.7±0.9	25.7±1.9	3.6±0.7	0.2±0.03	0.8±0.2	48.7±9.0	1.4±0.3	0.06±0.01	0.6±0.1	0.4±0.03	65.1±7.8	31.3±8.4
DOCA	Control (n=8)	18.8±0.5	27.9±0.9	19.3±1.2	0.7±0.05	1.5±0.2	35.7±3.3	14.5±0.7	0.5±0.03	1.4±0.1	0.4±0.03	ND	ND
	Ren-b^{Null} (n=6)	17.7±0.9	26.9±1.8	22.3±3.0	0.9±0.17	1.6±0.2	34.0±5.4	14.9±0.9	0.6±0.06	1.1±0.2	0.3±0.06	ND	ND
Stats		ND	2	1,2	1,2	1,2	ND	1,2	1,2	1,2	0	0	0

Fluid intake (using a two bottle choice of water and 0.15M saline), urine volume, sodium (UNaV) and potassium (UKV) excretion was measured in male and female mice at baseline and after DOCA-salt treatment. Males and females were combined as there was no difference in the level of fluid intake in preliminary analysis. The same mice were examined before and after DOCA-salt. The number of mice used in the study is indicated with the exception that UKC was measured in n=7 control mice. Statistics by Tukey analysis of 2-way Repeated Measures ANOVA: 0, no differences by genotype or DOCA; 1, effect of DOCA in control mice; 2, effect of DOCA within Ren-b^{Null} mice; 3, effect of genotype at baseline; 4, effect of genotype with DOCA; ND, not determined. Statistics for urine corticosterone and aldosterone was by unpaired T-test.

Table S2

Males

Tissue	Chow		HFD	
	Control (17)	Ren-b^{Null} (13)	Control (9)	Ren-b^{Null} (8)
Body mass (g)	32.9 ± 0.8	31.4 ± 0.9	48.3 ± 1.1 †	43.6 ± 1.2 ‡§
Heart (mg)	158 ± 4.7	159 ± 5.4	182 ± 6.4 †	180 ± 6.8 ‡
Liver (mg)	1359 ± 86	1335 ± 99	2449 ± 119 †	1683 ± 126 ‡§
Kidney (mg)	193 ± 11.8	204 ± 13.4	235 ± 16.2	204 ± 17.1
Interscapular BAT (mg)	95 ± 9.3	81 ± 10.6	206 ± 12.8 †	189 ± 13.6 ‡
Perigenital WAT (mg)	1010 ± 100	688 ± 115 *	1753 ± 138 †	1847 ± 146 ‡
Inguinal WAT (mg)	670 ± 81	522 ± 93	2783 ± 112 †	2107 ± 119 ‡§

Females

Tissue	Chow		HFD	
	Control (20)	Ren-b^{Null} (28)	Control (8)	Ren-b^{Null} (7)
Body mass (g)	25.3 ± 0.8	25.4 ± 0.7	33.8 ± 1.3 †	31.3 ± 1.4 ‡
Heart (mg)	124 ± 3.8	121 ± 3.2	129 ± 6.0	135 ± 6.4
Liver (mg)	1063 ± 42	1106 ± 36	1110 ± 67	1042 ± 72
Kidney (mg)	152 ± 5	149 ± 4.2	145 ± 7.9	164 ± 8.5
Interscapular BAT (mg)	63 ± 4.8	63 ± 4.1	97 ± 7.6 †	84 ± 8.2 ‡
Perigenital WAT (mg)	611 ± 103	689 ± 86	1842 ± 162 †	1353 ± 173 ‡§
Inguinal WAT (mg)	591 ± 94	641 ± 79	1784 ± 148 †	1463 ± 158 ‡

Table 2: Body and tissue mass. Body and tissue mass weighed in the sacrificed mice. (Upper table) Male Ren-b^{Null} mice exhibited normal body weight, but less perigenital white adipose tissue (WAT) compared to controls in normal chow. High-fat diet (HFD) increased the weight of body mass, heart, liver, brown adipose tissue (BAT), and WAT in both control and renin-b^{Null} mice. Within HFD group, however, the body weight, liver, and inguinal WAT were smaller in renin-b^{Null} mice compared to controls. (Lower table) In female mice, there was no significant difference between control and renin-b^{Null} mice fed normal chow. In HFD group, body weight was not different between genotypes, although perigenital WAT was smaller in renin-b^{Null} mice compared to controls. Two-way ANOVA was used. The sample numbers are indicated in the table. *, $P < 0.05$ Ren-b^{Null} chow vs control chow; †,$P < 0.05$ control HFD vs control chow; ‡,$P < 0.05$ Ren-b^{Null} HFD vs Ren-b^{Null} chow; §, $P < 0.05$ Ren-b^{Null} HFD vs control HFD. All data represent mean ± SEM.