Studies of Salt and Stress Sensitivity on Arterial Pressure in Renin-b Deficient Mice

Pablo Nakagawa¹, Javier Gomez¹, Ko-Ting Lu¹, Justin L. Grobe¹, and Curt D. Sigmund¹

¹Department of Physiology, Medical College of Wisconsin, Milwaukee

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Corresponding author:

Pablo Nakagawa, Ph.D.
Department of Physiology
Medical College of Wisconsin
8701 Watertown Plank Road
Milwaukee, WI 53226-0509
Email: pnakagawa@mcw.edu

Abstract

Excessive sodium intake is known to increase the risk for hypertension, heart disease, and stroke. Individuals who are more susceptible to the effects of high salt are at higher risk for cardiovascular diseases even independent of their blood pressure status. Local activation of the renin-angiotensin system (RAS) in the brain, among other mechanisms, has been hypothesized to play a key role in contributing to salt balance. We have previously shown that deletion of the alternative renin isoform termed renin-b disinhibits the classical renin-a encoding preprorenin in the brain resulting in elevated brain RAS activity. Thus, we hypothesized that renin-b deficiency results in higher susceptibility to salt-induced elevation in blood pressure. Telemetry implanted Ren-b^{Null} and wildtype littermate mice were first offered a low salt diet for a week and subsequently a high salt diet for another week. A high salt diet induced a mild blood pressure elevation in both Ren-b^{Null} and wildtype mice, but mice lacking renin-b did not exhibit an exaggerated pressor response. When renin-b deficient mice were exposed to a high salt diet for a longer duration (4 weeks), was a trend for increased myocardial enlargement in Ren-b^{Null} mice when compared with control mice. Multiple studies have also demonstrated the association of chronic and acute environmental stress with hypertension. Activation of the RAS in the rostral ventrolateral medulla and the hypothalamus is required for stress-induced hypertension. Thus, we next questioned whether the lack of renin-b would result in exacerbated response to an acute restraint-stress. Wildtype and Ren-b^{Null} mice equally exhibited elevated blood pressure in response to restraint-stress, which was similar in mice fed either a low or high salt diet. These studies highlight a complex mechanism that masks/unmasks roles for renin-b in cardiovascular physiology.

Introduction

High blood pressure is a leading cause of complications from heart disease, stroke, and kidney disease. Importantly, it has been shown that in both normotensive and hypertensive subjects, increased sensitivity to sodium increases the risk for cardiovascular mortality and morbidity [1]. The increased prevalence of hypertension in the last century has been attributed in part to the higher consumption of dietary salt [2]. Moreover, there is strong evidence in animal models and human for a causative role of dietary sodium intake in the development of hypertension [2-4]. Dietary sodium reduction in combination with the DASH (dietary approaches to stop hypertension) diet is a powerful approach to control blood pressure in hypertensive patients [5, 6].

According to Guyton and Coleman's hypothesis, salt-sensitive hypertension can develop when there is an impaired excretory ability of the kidney that shifts the relation between sodium excretion and arterial pressure toward higher values [7]. Recently, Fujita et al. identified a key mechanism implicating renal β 2-sympathetic stimulation contributing to the development of salt-sensitive hypertension [8]. Numerous studies have identified neurogenic actions of elevated salt intake. For instance, elevated dietary sodium intake sensitizes sympathetic neurons located in the in response to a stimulus [9]. In addition to the effects on arterial pressure, high salt diet can also adversely affect several organs including the vasculature, heart, and kidneys. Interestingly, some of these effects appear to be independent of blood pressure status [10].

46 The renin-angiotensin system (RAS) is recognized as a powerful circulating hormone
 47 system regulating blood pressure and sodium homeostasis. Although high salt intake
 48 suppresses the circulating classical RAS, the deterioration of renal function in sodium
 49 loaded animals can be attenuated by the blockade of the RAS [11]. Thus, it has been
 50 proposed that activation of a locally activated RAS within tissues, which acts
 51 independently of the circulating RAS, can be of major importance in the regulation of the
 52 angiotensin (Ang) II generation in the kidney, brain, vasculature, and heart [12-16]. Renin
 53 is the rate-limiting step of Ang II synthesis and exists in at least two isoforms. The classical
 54 renin isoform encoding preprorenin termed “renin-a” is primarily expressed by the
 55 juxtaglomerular cells of the kidney and is released into the circulatory system in response
 56 to a number of stimuli. On the contrary, the novel alternative renin isoform, termed “renin-
 57 b”, is mainly expressed in the brain, heart, and adrenal gland [17-19]. Current evidence
 58 suggests that renin-b is unlikely to be secreted as it lacks a secretory peptide. Our
 59 previous data suggest that renin-b maintains brain RAS activity at normal physiological
 60 levels by suppressing the classical renin isoform and that the genetic removal of renin-b
 61 triggers a mild increase in blood pressure and sympathetic nerve activity [20-22]. The
 62 sustained suppression of the brain RAS by renin-b in healthy individuals may have
 63 important implications in maintaining the cardiovascular homeostasis during different
 64 hypertension-triggering stimuli such as high salt diet and other environmental stressors.
 65 Blood pressure elevation induced by the exposure to physical stress can be ameliorated
 66 when the brain RAS is suppressed [23, 24]. Given the rationale that hypertension-prone
 67 animal models exhibit a greater increase in blood pressure and heart rate in response to
 68 restraint-stress and air-jet stress [25, 26] we hypothesized that mice lacking renin-b would

69 be more responsive to an acute environmental stressor as consequence of brain RAS
70 disinhibition.

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Methods

Animals: All experiments were conducted using mice lacking exon 1b of the renin gene on the C57BL/6 genetic background as previously described [20]. Experimental animals were generated by intercrossing heterozygotes to generate homozygous control wildtypes (WT) and homozygous Ren-b^{Null} mice. Only males were used except where indicated. Males were preferred in this study because we previously observed that Ren-b^{Null} males exhibit an exacerbated phenotype compared to females [21]. A room with low levels of noise and traffic was designated for these experiments. Animals received standard laboratory chow (NIH-31 Modified Open Formula Mouse/Rat sterilizable diet; Teklad catalog # 7913; Harlan Teklad) until exposure to low and high salt diets. All experiments were conducted with the approval of the University of Iowa and Medical College of Wisconsin Animal Care and Use Committees and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Blood pressure measurements: Twenty-four-hour systolic, diastolic, and mean blood pressures and heart rate telemetric data were acquired by radiotelemetry. Telemetry implantation surgeries and telemetric data acquisition were performed by a surgeon who was blinded to genotype. Anesthesia was induced with ketamine (90 mg/kg i.p.)/xylazine (5 mg/kg i.p.) to implant the telemetry catheters. Then, a 1-2 cm incision was made to expose the internal carotid artery. A TA11PA-C10 radiotelemetry catheter (DSI, New Brighton, MN) was inserted in the left carotid artery and advanced into the transverse aorta. The catheter was secured with three sutures and the incision was sutured with 6-

0 surgical silk. A triple antibiotic ointment, Vetropolycin (Dechra Pharmaceuticals PLC, Northwich, United Kingdom), was applied to the wound. Flunixin (2.5 mg/kg, i.p.) was immediately applied after the surgery and again 24 hours later. Then, the animals were housed in a cage placed on a heating pad overnight with easy access to food and water. Once awake from the insertion of the radiotelemetry implant procedure, the animals were housed individually in a regular cage placed on top of the telemetry receiver plate with free access to water and a standard chow diet containing 0.3% Sodium. Baseline blood pressure and heart rate data were acquired for 3 days after 10 days of post-surgical recovery. Blood pressure and heart rate data were recorded for 10 seconds every 5 minutes for 24 hours and averaged daily, hourly, and during the light (5 AM to 5 PM) and dark phase (5 PM to 5 AM).

Assessment of salt sensitivity:

Experiment 1: Telemetry implanted mice underwent 3-day baseline blood pressure and heart rate data acquisition under a normal salt diet containing 0.8% NaCl (NIH-31 Modified Open Formula Mouse/Rat sterilizable diet; Harlan Teklad). The standard diet containing normal salt was then replaced with a salt-deficient chow diet containing 0.01-0.02% sodium (TD08290, Envigo, Indianapolis, IN). Data were acquired for 7 days on a low salt diet. Then, the diet was switched to a high salt chow containing 4% NaCl (TD03095, Envigo, Indianapolis, IN) and data were acquired for 7 additional days (Figure 1).

Experiment 2: Renal concentrating response to acute hypo and hyperosmotic load was assessed in a cohort of male and female WT, heterozygous, and Ren-b^{Null} mice. Urine osmolality in response to acute intraperitoneal injections of 0.5 milliliters of a hypotonic or hypertonic saline solution was assessed using a freezing point depression multi-sample osmometer (OsmoPro, Advanced Instruments, Norwood, MA).

Experiment 3: A separate cohort of mice without telemeters were placed in metabolic cages for urine collection at baseline, under a standard chow diet (0.8% NaCl), and after 3 weeks on a high salt diet (4% NaCl). Food and drinking intake, urinary volume, and protein excretion were assessed. Protein concentration was measured using a commercially available protein determination kit from Thermo Scientific (catalog # 23225), which is based on the bicinchoninic acid method. By week 4, blood samples were obtained from vena cava using a heparinized syringe for measurement of plasma renin. Subsequently, mice were sacrificed, and kidneys, heart, liver, spleen, and lungs were collected and weighed.

Responses to restraint stress in mice fed high or low salt diets:

Experiment 4: Telemetry implanted mice tested for salt-sensitivity in experiment 1 were subsequently subjected to acute experiments to assess sensitization to restraint-stress (Figure 1). First, we evaluated the cardiovascular responses to an acute immobilization protocol. WT and Ren-b^{Null} mice fed high salt diet were placed for 10 minutes in a restrainer, while telemetric blood pressure and heart rates were monitored. Then, the diet was switched back to a low salt diet and the same procedure was performed 14 days

later. Baseline telemetric blood pressure and heart rate were acquired for 10 minutes before mice were placed in the restrainer. Once in the restrainer, data were acquired for 10 additional minutes. A separate cohort of males and females fed with a normal salt diet was used to study the metabolic responses to restraint-stress. Blood glucose was measured at baseline before restraint-stress and induced a restrain-stress for 30 minutes. Finally, mice were released to the home cage and blood glucose was measured again during recovery. Every 15 minutes 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.). Mice were sacrificed by decapitation at 30 minutes post-recovery phase and blood was collected for renin and glucocorticoid determinations.

Plasma renin: Heparinized blood was collected in 1.5 ml tubes from the inferior vena cava in anesthetized mice or trunk after decapitation. Blood samples were centrifuged at 4000 rpm for 2 min at 4° C to isolate plasma. Plasma renin was measured using a commercially available Sandwich-based ELISA kit (Raybiotech, Peachtree Corners, GA) following the manufacturer's recommended instructions. Samples were processed and analyzed by a blinded operator.

Statistical analysis: All data are presented as mean±SEM. Data were analyzed using GraphPad Prism software. All telemetric data, parameters derived from metabolic cage studies and osmotic challenge tests, plasma glucose, renin and corticosterone measurements were analyzed using two-way ANOVA with repeated measures, followed by Sidak's multiple comparison procedures. Tissue weights were analyzed using

164 unpaired t-test. A *P value* of less than 0.05 was considered significant. Individual data
165 points were plotted in dot/whisker plots if possible.

Results

Retrospective analysis of the arterial blood pressure in Ren-b^{Null} cohorts

Retrospective analysis of blood pressure in five cohorts of Ren-b^{Null} and WT mice (listed in order of experimentation) indicated that mice lacking Ren-b exhibit a high degree of variability in blood pressure (range = 27.7 mmHg, Figure 2). The range of blood pressure in control mice was much smaller (5.5 mmHg). We hypothesized that these differences might be attributed to environmental factors as a review of conditions under which the cohorts were studied revealed that cohorts 1, 2, 3, and 4 were housed in a large room with an elevated level of noise and traffic (data collected between March 2014 and October 2015 [20]), while mice in cohort 5 were housed in a smaller quiet animal facility located in a different building (data collected from 2/2017 to 11/2017 [27]). Cohorts 2 and 4 were exposed to an additional stress as they were subjected to a surgical implantation of a stainless-steel intracerebroventricular cannula for subsequent acute administration of drugs. These data were previously reported [20, 27]. Cohorts 3 and 4 were subjected to extensive animal handling during experiments. Noise, post-surgical stress and animal handling appeared associated with an exacerbated blood pressure increase in Ren-b^{Null} mice. Therefore, the aim of this study was to elucidate whether the manifestation of cardiovascular and renal phenotypes in Ren-b^{Null} mice are due to increased sensitivity of these animals to external stressors such as environmental stimuli (i.e., noise, diet, and other psychological stresses such as restraint or handling).

Effect of high salt diet on blood pressure in Ren-b^{Null} mice.

Wildtype and Ren-b^{Null} mice fed a normal salt diet were instrumented with telemetric implants and allowed to recover for 10 days in a quiet environment. As shown in Figure 3, baseline blood pressure was first measured for 3 days under a normal salt diet containing 0.3% sodium. Then, the diet was replaced from normal to a low salt diet containing chow (Na 0.01-0.02%) and blood pressure recordings continued for 7 additional days. Finally, mice were fed high-salt diet chow (NaCl 4%) for the last 7 days. The low salt diet had no effects on systolic blood pressure, diastolic blood pressure, mean blood pressure or heart rate compared with the baseline diet (Figure 3). Blood pressure was increased in both wildtype and Ren-b^{Null} mice in response to the high salt diet when compared with the baseline diet ($P<0.05$) or low salt diet ($P<0.05$). Heart rate was not altered. Contrary to our hypothesis, there was no difference in blood pressure or heart rate comparing wildtype and Ren-b^{Null} mice in any diet.

To evaluate the capacity of Ren-b^{Null} mice to excrete a sodium load, mice were injected with either intraperitoneal hypo- or hyperosmotic NaCl solutions (0.5 ml of 0.03 and 0.3 mol/L NaCl, respectively). As expected, hyper-osmotic saline increased urinary osmolality compared with hypoosmotic saline injection. However, there was no significant difference in the urinary osmolality between wildtype, heterozygous, and Ren-b^{Null} mice (Figure 4).

A cohort of Ren-b^{Null} mice without telemetry implants was placed in metabolic cages before and after 3 weeks on a high salt diet. One animal was excluded due to malocclusion. When compared with parameters at baseline, both WT and Ren-b^{Null} mice fed a high salt diet for 3 weeks showed an increase in body weight and urine volume and

a decrease in food intake and feces excretion (Table 1). Interestingly, an early cohort of Ren-b^{Null} mice exhibited higher urinary protein excretion compared with wildtype mice at baseline. The level of protein excretion was maintained in Ren-b^{Null} mice after high salt diet. On the contrary, high salt diet increased urinary protein excretion in WT mice. Consistent with our prior studies on normal salt diet fed mice, Ren-b^{Null} mice fed a high salt diet showed a trend toward decrease in plasma renin ($p=0.094$) [27]. Ren-b^{Null} mice on high salt exhibited a trend toward increased heart weight normalized by body weight compared with the WT on a high salt diet ($p=0.08$) (Table 2). No differences in body weight (WT = 28.36 ± 1.16 g vs Ren-b^{Null} = 29.84 ± 1.45 g), kidney weight, liver weight, spleen weight, and lung weight were found among groups.

Responses to acute restraint-stress

Since there was no difference in blood pressure in response to the high salt diet, the same animals were further challenged with an acute restraint-stress. After 10 minutes of baseline recording, mice were quickly placed in an acrylic restrainer, and blood pressure and heart rate were recorded for additional 10 minutes. Restraint-stress induced a ~25 mmHg blood pressure elevation in both WT and Ren-b^{Null} mice which was associated with a robust tachycardic response (~200 bpm). No difference in the pressor responses to restraint-stress was observed in WT compared with Ren-b^{Null} mice (Figure 5A). Interestingly, Ren-b^{Null} exhibited elevated heart rate ($P_{\text{genotype}} < 0.05$), which was more prominent at baseline as it achieved a similar increase compared with WT when exposed to restraint-stress. The individual values of blood pressure and heart rate before and during restraint-stress were plotted separately (Figure 5B). After the acute stress

challenge was completed, the mice were shifted back to low salt diet for 2 weeks. Similarly, restraint-stress induced an increase in both blood pressure and heart rate in both groups, but no difference between WT and Ren-b^{Null} was observed (Figure 6).

Previous cohorts of Ren-b^{Null} mice exhibited resistance to body weight gain in response to high fat diet, which was attributed to elevated resting metabolic rate and sympathetic outflow to the brown adipose tissue [21]. Since restraint-stress induces hyperglycemia through the central catecholaminergic neuronal system located in the rostral ventrolateral medulla [28], we evaluated in a separate cohort of male and female mice whether Ren-b^{Null} mice exhibit differences in the blood glucose response to restraint-stress. Blood glucose levels were measure in 15 minute-intervals before, during, and after mice were restrained. Control mice were kept in their home cages. Restraint-stress induced a significant increase in blood glucose in both wildtype and Ren-b^{Null} mice (P=0.0002). However, there was no difference between genotypes (Figure 7A). Plasma renin and glucocorticoids were measured 30 minutes after mice were released from the restraint. As above, unrestrained Ren-b^{Null} mice exhibited a trend towards a decrease in plasma renin, but this difference was not observed in restrained mice (Figure 7B). No differences in plasma glucocorticoids were observed between animals at the end of the protocol (Figure 7C).

Discussion

The main finding of this study is that in a mouse model lacking renin-b, there were significant differences in blood pressure among study cohorts which cannot be attributed to changes in dietary sodium intake nor psychological stress. First, we performed retrospective analyses of the blood pressures in five different Ren-b^{Null} cohorts studied over a 3-year period from 2014 to 2017. Interestingly, we observed that the cohorts that exhibited the highest blood pressure were housed and manipulated under conditions that might trigger a stress response. Second, since we have previously observed that ablation of renin-b results in elevated renal sympathetic nerve activity we questioned whether mice lacking renin-b would develop impaired salt handling and thus salt sensitivity. Finally, Ren-b^{Null} mice on high or low salt diet were further challenged to acute restraint-induced stress. Despite these stimuli, Ren-b^{Null} mice did not exhibit a clear difference in blood pressure compared with control littermates.

We previously identified that in models of salt-dependent hypertension such as deoxycorticosterone-salt hypertension there is a downregulation of renin-b expression in the brain with a concomitant elevation of the classical renin isoform (renin-a) encoding preprorenin [29]. Similarly, genetic ablation of renin-b resulted in an augmented expression of renin-a in several brain nuclei including the rostral ventrolateral medulla [20]. We reported that disinhibition of the brain RAS due to renin-b genetic deletion results in a mild elevation of blood pressure, which was accompanied by an elevated renal sympathetic nerve activity. However, after successive rounds of backcross breeding, the mice developed a milder phenotype with no apparent blood pressure elevation [27].

Surprisingly, despite the loss of an apparent blood pressure phenotype, normotensive Ren-b^{Null} mice exhibited an exaggerated decrease in plasma renin and an increased susceptibility to end organ damage caused by angiotensin II which would be consistent with hypertension [27]. The decrease in plasma renin observed in previously reported cohorts was replicated herein. Here we considered that environmental or dietary factors might be involved in these differences between cohorts.

To test whether ablation of Ren-b results in salt-sensitivity, Renin-b knockout mice and wildtype littermates were first fed a low salt diet for 7 days and subsequently switched to a high salt diet for the next 7 days. A diet high in salt induced a modest increase in blood pressure in both wildtype and renin-b knockout mice. It was recently demonstrated that the C57BL6/J strain is salt-sensitive in response to overactivation of the sympathetic nervous system [30], but this remains controversial as there are other reports indicating that C57BL6/J are salt resistant [31, 32]. Contrary to our prediction, renin-b lacking mice did not exhibit an augmented pressor response to salt. Therefore, we concluded renin-b deficiency is insufficient to induce an increased sensitivity to a pressor response to high salt diet.

Several studies indicate that elevated sodium intake can lead to functional and structural cardiac remodeling independent of changes in blood pressure [10, 33]. Here, we observed that Ren-b^{Null} mice exhibit a trend towards increase in heart weight when fed a high salt diet. This observation is not surprising because we showed that Ang-II infusion also induced elevated heart weight in Ren-b^{Null} mice which was accompanied by induction

of pro-inflammatory and pro-fibrotic genes in the myocardium [27]. Thus, Ren-b^{Null} mice retain a susceptibility to what would be considered hypertensive end-organ damage. Studies from Jörg Peters's laboratory have evidenced that cardiac insult triggers renin-b upregulation in the myocardium [34]. Furthermore, myocardial renin-b overexpression results in cardioprotection against ischemic and hypoglycemic conditions [35, 36]. Future studies using conditional and tissue-specific models will be required to elucidate the distinct functions of renin-b in different cells including cardiomyocytes.

Given the milder phenotype that is observed in the current cohorts of renin-b knockout mice, we hypothesized that ablation of renin-b leads to an enhanced susceptibility to hypertension-eliciting psychological and physical stressors. Under normal conditions, the activity of the brain RAS is relatively low because it is tonically suppressed by renin-b among other factors. However, it has been shown that animals preconditioned to different stressful conditions can lead to brain RAS disinhibition, a condition that can be recapitulated in the Ren-b^{Null} model. Importantly, disinhibition of the brain RAS in response to these environmental factors results in a higher predisposition to hypertension due to enhanced sensitization to a subsequent hypertensive stimulus including Ang-II [37, 38]. Therefore, in this current study, we investigated whether renin-b lacking mice are sensitized to an acute restraint-stress induced pressor and metabolic responses. Restraint-stress induced an instantaneous pressor and tachycardiac response in both wildtype and Ren-b^{Null} mice. However, there was no significant difference in the magnitude of the response and the timing to maximal blood pressure between them. This suggests that ablation of renin-b does not cause a significant susceptibility to this particular

acute stressor.

The main limitation of this study is that the animals were exposed to a single acute exposure to a particular stress induced by immobilization. We observed that restraint induces a dramatic response evidenced by a marked and rapid increase in blood pressure and heart rate. Thus, it is possible that the maximal nature of this response may have caused us to not observe an increased response in Ren-b^{Null} mice to a submaximal stress stimulus. It also remains unclear whether a chronic repetitive daily exposure to not only restraint-stress, but also different types of stressors such as noise, wet cage, and vibrations would unmask the cardiovascular and other phenotypes in Ren-b^{Null} mice. We recognize that we are limited to recapitulate exactly the environmental conditions that once caused elevated blood pressure in this model, but we are certainly confident that Ren-b^{Null} mice are resistant to changes in sodium intake and an acute stressor in our current conditions. We cannot rule out a possibility that a genetic drift occurred in our colony after several breeding cycles. Thus, ongoing studies where Ren-b^{Null} mice are backcrossed to a different genetic background will be useful to determine whether the discrepancies in blood pressure occurred due to spontaneous changes in the DNA along intense inbreeding protocols. Finally, other variables such as the microbiome, variations in temperature and humidity, as well as variations between batches of bedding and chow are difficult to control, but they must be recognized as an important source of variability between experimental cohorts.

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Disclosures

C.D. Sigmund is a member of the Scientific Advisory Board for Ionis Pharmaceuticals. His contributions to that board are unrelated to the content of this article. The other authors report no conflicts.

Figure Legends

Figure 1: Experimental Protocol.

The assessment of salt sensitivity (Experiment 1) and susceptibility to restraint-stress (Experiment 4) were performed in the same animals. Male Ren-b^{Null} or wildtype (WT) littermate controls were instrumented with a radiotelemetry catheter and housed in a quiet environment for blood pressure and heart rate measurements. In experiment 1, baseline arterial blood pressure and heart rate were recorded for 3 days under a standard salt diet (SD, 0.8 % NaCl). Subsequently, the diet was switched to a chow containing low sodium (LS, 0.02 % NaCl), and data were acquired for 7 additional days. Finally, the diet was switched from low to high salt containing diet (HSD, 4 % NaCl), and data were acquired for 7 additional days. To study responses to acute restraint stress in both HSD and LSD, the animals were continued on HSD for an additional week. By the end of the second week on HSD, mice were placed to an acrylic restrainer to assess the acute cardiovascular responses to restraint-stress. Next, mice were fed LSD for two weeks and the acute responses to restraint-stress were reassessed again.

Figure 2: Cohort Analysis of Arterial Blood Pressure.

Reanalysis and comparison of the telemetric blood pressure in 5 independent cohorts of Ren-b^{Null} and wildtype (WT) mice. Summary of the systolic blood pressure (SBP) during the light cycle is shown. This data was previously reported in references [20] and [27].
*P<0.05 vs WT by unpaired t-test.

Figure 3. Blood Pressure Response to Salt.

Effect of salt intake on systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) in male Ren-b^{Null} mice and wildtype littermate controls (WT) housed in a quiet environment. Following a 3-day baseline radiotelemetric data collection period, the diet was switched from a standard diet to a chow containing 0.02% NaCl (TD08290, LSD). Data were acquired for 7 days. Subsequently, mice were challenged with a diet containing 4% NaCl (TD03095, HSD), and data were collected for 7 additional days. Twenty-four-hour averaged SBP (left), DBP (center), and HR (right) were expressed as mean $\pm$ standard error of the mean (top panel). For purposes of clarity and transparency, the individual points for weekly averaged SBP, DBP, and HR data were also plotted (bottom panel). Data were analyzed by two-way ANOVA with Sidak's multiple comparisons procedure. Adjusted $p < 0.05$ was considered significant. * $p < 0.05$ vs WT at baseline, # $p < 0.05$ vs Ren-b^{Null} at baseline, Φ $p < 0.05$ vs WT fed LSD, Ψ $p < 0.05$ vs Ren-b^{Null} fed LSD.

Figure 4: Renal Function.

Urine concentrating capacity was assessed in male and female wildtype (Ren-b^{+/+}), heterozygous Ren-b knockout (Ren-b^{+/-}) and homozygous Ren-b knockout mice (Ren-b^{-/-}). Urine was first collected for 60 minutes post-hypotonic saline challenge (500 microliters of 0.03 mol/L NaCl; intraperitoneal). Subsequently, the same procedure was performed for a hypertonic saline challenge (500 microliters of 0.3 mol/L NaCl; intraperitoneal). * $p < 0.05$ vs same genetic group on hypotonic challenge.

Figure 5: Blood Pressure Response to Restraint Stress (HSD).

Acute responses to restraint-induced blood pressure and tachycardia in male Ren-b^{Null} mice and wildtype littermate controls (WT) fed high salt diet. First, telemetric recordings were obtained from high salt diet-fed mice in their home cages at baseline. Following 10 minutes of recording, mice were immediately inserted into an acrylic restrainer and telemetric data were acquired for 10 minutes. A) One-minute recording was averaged and data were expressed as mean $\pm$ standard error of the mean. B) For purposes of clarity and transparency the individual 10 minutes systolic blood pressure and heart rate average of each animal under baseline and restrained conditions was plotted. Data were analyzed by two-way ANOVA with Sidak's multiple comparisons procedure. Adjusted p<0.05 was considered significant.

Figure 6: Blood Pressure Response to Restraint Stress (LSD).

Acute responses to restraint-induced blood pressure and tachycardia in male Ren-b^{Null} mice and wildtype littermate controls (WT) fed low salt diet. First, telemetric recordings were obtained from low salt diet-fed mice in their home cages at baseline. Following 10 minutes of recording, mice were immediately inserted into an acrylic restrainer and telemetric data were acquired for 10 minutes. A) One-minute recording was averaged and data were expressed as mean $\pm$ standard error of the mean. B) For purposes of clarity and transparency the individual 10 minutes systolic blood pressure and heart rate average of each animal under baseline and restrained conditions was plotted. Data were analyzed by two-way ANOVA with Sidak's multiple comparisons procedure. Adjusted p<0.05 was considered significant.

439 Figure 7: A) Blood glucose was determined in male and female Ren-b^{Null} and wildtype
 440 littermates subjected to restraint stress or kept in their home cages. The area under the
 441 curve (AUC) was calculated (right). Plasma renin (B) and corticosterone (C) were
 442 determined at 30 minutes post-recovery phase. *P<0.05 vs unrestrained WT and #P<0.05
 443 vs unrestrained Ren-b^{Null}.

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	Baseline		HSD		p-value		
	WT (n=5)	Ren-b ^{Null} (n=5)	WT (n=5-6)	Ren-b ^{Null} (n=5-6)	Timepoint	Genotype	Interaction
Body weight (g)	27.26±0.60	26.54±0.75	28.10±0.48	27.60±0.84	0.002	0.533	0.620
Food intake (g/day)	2.63±0.23	2.90±0.33	2.16±0.19	2.10±0.21*	0.008	0.744	0.383
Water intake (ml/day)	2.99±0.30	3.05±0.34	3.86±0.39	3.24±0.38	0.109	0.511	0.280
Feces weight (g/day)	0.70±0.09	0.85±0.12	0.44±0.03*	0.49±0.07*	0.001	0.374	0.488
Urine volume (ml/day)	0.65±0.16	0.61±0.07	1.13±0.12	1.39±0.34	0.015	0.604	0.492
Urinary protein excretion (mg/day)	11.74±5.62	51.66±9.70 [#]	31.61±7.37	44.12±4.52	0.374	0.009	0.070
Plasma renin (ng/ml)			44.8±12.8	24.3±7.0	0.094		

Table 1: Male Wildtype (WT) and Ren-b^{Null} mice were housed in metabolic cages to measure food intake, feces weight, water intake, urine volume, and urinary protein excretion at baseline and after 4 weeks on high salt diet (HSD, 4% NaCl). At the end of the protocol renin was measured from plasma samples. Except plasma renin the data were analyzed by two-way ANOVA with Sidak's multiple comparisons procedure. Plasma renin data were analyzed by unpaired t-test. *p<0.05 vs same genotype at baseline and [#]p<0.05 vs WT at same timepoint.

	Tissue weight (g)		Tissue weight/body weight (mg/g)	
	WT+HSD (n=6)	Ren-b ^{Null} +HSD (n=5)	WT+HSD (n=6)	Ren-b ^{Null} +HSD (n=5)
Heart	0.125±0.003	0.130±0.003	4.26±0.25	4.72±0.10
Lungs	0.157±0.006	0.150±0.010	5.34±0.17	5.43±0.30
Kidneys	0.177±0.009	0.173±0.006	6.01±0.21	6.27±0.06
Liver	1.217±0.084	1.256±0.066	41.48±2.96	45.55±2.24
Spleen	0.085±0.004	0.080±0.003	2.93±0.25	2.90±0.10

Table 2: Body weight (left column) and tissue weights normalized by body weight (right column) in male Ren-b^{Null} mice and wildtype littermate controls (WT) fed a high salt diet (HSD, 4% NaCl) for 4 weeks. An unpaired t-test was used for data analysis.

Experimental design

Experimental animals:

- WT littermate control
- Ren-b^{Null}

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7