Renal sympathetic nerve activity regulates cardiovascular energy expenditure in rats fed high salt

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Abstract

We recently reported that 4% high salt diet + saline for drinking (HS+saline) led to a catabolic state, a reduced heart rate, and suppression of cardiovascular energy expenditure in mice. We suggested that HS+saline reduced heart rate via the suppression of the sympathetic nervous system to compensate for the high-salt intake-induced catabolic state. To test this hypothesis, we directly measured renal sympathetic nerve activity (RSNA) using a radiotelemetry system in conscious Sprague-Dawley (SD) rats. We confirmed that HS+saline induced a catabolic state. HS+saline decreased heart rate in association with a reduction in RSNA in SD rats. By contrast, Dahl salt-sensitive (DSS) rats exhibited no change in heart rate and increased their RSNA during HS+saline. Renal denervation significantly decreased heart rate and attenuated the catabolic state independent of blood pressure in DSS rats fed HS, suggesting that salt-sensitive animals could not decrease cardiovascular energy consumption due to the abnormal renal sympathetic nerve activation during high salt intake. These findings support the hypothesis that RSNA mediates heart rate during high salt intake in SD rats. However, the insensitivity of heart rate and the enhanced RSNA in DSS rats could be an additional critical diagnostic factor for salt-sensitive hypertension. Renal denervation may benefit salt-sensitive hypertension by reducing effects on catabolism and cardiovascular energy expenditure.

Keywords: Salt, renal sympathetic nervous system, salt-sensitive hypertension, heart rate

Introduction

Sodium is the main extracellular ion, and the steady-state concept of Na^+ homeostasis states that sodium regulation is responsible for extracellular volume and water content [1, 2]. Sodium and anions are osmolytes that draw in and retain water in the body. Salt is excreted into the urine and the resulting increase in urinary osmolytes (Na^+ and Cl^-) generates an osmotic driving force for water excretion. In addition, eating large amounts of salt increases thirst. According to current notions, a high-salt intake increases water intake and urine volume to maintain the sodium and water balance [3-5]. These conclusions were largely drawn from studies at extremes of salt intake. We examined ultra-long-term sodium and water balance in healthy humans in response to various normal-range salt intakes (6, 9 and 12 g/day) under metabolic ward conditions [3]. The results of the study revealed that a 6 g/day increase in daily salt intake did not affect urine volume and significantly decreased drinking in healthy subjects [3]. It was also shown that urea-driven body water conservation via a hepato-renal-muscular axis facilitated water balance during a high salt intake [3, 6]. On the basis of our findings, we suggested some alternative explanations regarding water balance in response to salt intake as follows: During a high-salt intake (i) urea-driven water reabsorption/urine Na^+ concentration mechanism is effective in the kidney to excrete the salt load with a minimum of water losses. During high salt intake (ii) the urea-producing enzyme, arginase, drives urea production in the liver, which consumes ATP and amino acids and induces a catabolic state. The increased catabolism is compensated by either increased food intake or by energy and nitrogen transfer from skeletal muscle. In the latter case; (iii) nitrogen and energy transfer from skeletal muscle to the liver via the alanine-glucose-shuttle predisposes to body weight loss due to muscle wasting [3, 6]. The urea-driven

renal free water reabsorption counterbalances Na^+ and Cl^- excretion-driven osmotic diuresis [3, 6]. Therefore, a high salt diet did not increase urine volume in humans [3]. In addition, the energy-intensive nature of hepatic urea production led to a catabolic state, predisposing to an increase in endogenous metabolic water production. The endogenous water may explain the reduced fluid intake that we observed in humans [3]. These findings suggest that the natriuretic-ureotelic regulation of body fluid homeostasis could be an additional critical feature of a high-salt intake, which leads to energy deficit and induces a catabolic state [3, 6].

In our mouse model, the salt-driven catabolic state led to changes in not only energy metabolism in the liver and muscle, but also altered cardiovascular function [6]. High-salt-fed mice exhibit reduced cardiovascular energy expenditure via a decrease in heart rate, implying that energy was saved for water conservation [6]. Since higher heart rates and a catabolic state are major risk factors of cardiovascular diseases [7-10], the slower heart rate and reduced cardiovascular energy expenditure may be important responses to high-salt intake for preventing cardiovascular events. We elected to investigate these responses further. Since the sympathetic nervous system is a master regulator of cardiovascular function [11], we measured renal sympathetic nerve activity (RSNA) directly in conscious rats using a radiotelemetry system. We used Sprague-Dawley (SD) strain as a general control model of rat in the present study, which is a healthy strain and widely used for investigating a physiological response. In separate experiments, we also studied Dahl salt-sensitive (DSS) rats as a salt-sensitive hypertension model and identified significant differences in the responses of RSNA.

Methods

Animal study

The Animal Research Committee of Kagawa University and Osaka General City Hospital approved all experimental protocols and procedures (No. 15037 & 18627). Male SD (Slc:SD) and DSS (DIS/EiS) rats (Japan SLC Inc, Shizuoka, Japan) were used, and the animals were housed under controlled temperature (24±2°C) and humidity (55±5%) on a 12-h light/dark cycle. A 0.2% NaCl normal salt diet (NS) and 4% NaCl high salt diet (HS) were used (Oriental Yeast Co, Tokyo, Japan, Supplementary table 1) and fed *ad libitum*.

Experimental protocol 1: Male, 6-week-old SD rats in the HS or NS groups were provided free access with or without 0.9% NaCl water to drink for 6 weeks (n=6 or 7 per group). Daily food intake and body weight were measured, urine samples were collected before the sacrifice and the rats were sacrificed 6 weeks following treatment.

Experimental protocol 2: A radiotelemetry system was implanted into SD, DSS, and renal-denervated DSS rats (250–300 g body weight), and arterial blood pressure, heart rate and RSNA were measured in conscious rats. A sham operation or renal denervation (RDX) and a radiotelemetry insertion were performed in one surgical procedure. The rats were fed with NS, HS, and HS+saline for 4 days in this order 10–14 days following surgery.

Experimental protocol 3: Male, 4-week-old DSS rats were divided into three groups: i) sham operation + NS, ii) sham operation + HS+saline, and iii) RDX + HS+saline (n=6 per group) in which a sham operation or RDX was performed. At 2 weeks post-surgery, the rats were fed the respective diets. Daily food intake and body weight were measured,

urine samples were collected before the sacrifice and the rats were sacrificed 6 weeks following treatment. Systolic blood pressure was also measured once per week by a tail-cuff method during the experiment.

Blood Pressure Measurement

A tail-cuff plethysmograph (BP-98A; Softron Co, Tokyo, Japan) was used in experimental protocols 1 and 3, and a radiotelemetry system (Data Sciences International (DSI), St Paul, MN, USA) was used in experimental protocol 2, as described previously [12]. A radiofrequency transmitter (PA-C40; DSI) and a receiver (RPC-1; DSI) were used to measure blood pressure and heart rate in renal-denervated rats. Data were collected and analyzed using Dataquest ART version 4.3 (DSI).

Urine collection and urinary sodium measurement

Twenty four-h urine volume and fluid intake were measured in the experiment-1and -3. All rats underwent a 12-h acclimatization period in metabolic cages prior to urine collection. Urinary sodium excretion (UNaV) was measured by using an automated analyzer (7020-Automatic Analyzer; Hitachi High-Technologies, Japan) as described previously [13].

Renal noradrenaline content measurement

Renal noradrenaline content was measured as described previously [14]. Briefly, 100 mg of the renal cortex tissue was collected and homogenized in 500 μL of 0.2 M perchloric acid, and then centrifuged (0°C, 20,000 g, 15 min). Following centrifugation, the supernatant was filtered using a regenerated cellulose membrane (Minisart RC4, Sartorius,

Japan) and subjected to high-performance liquid chromatography for the measurement of noradrenaline content.

Renal Denervation

Bilateral RDX was performed, as described previously [13, 15]. Briefly, all detectable renal nerves around the renal arteries and veins were cut, following which the vessels were painted with a solution of 10% phenol in ethanol. In the present study, the efficiency of RDX was confirmed by measuring renal noradrenaline content at 4 weeks post-surgery in experimental protocol-2 (163 ± 19 ng/g tissue versus 7.6 ± 4.3 ng/g tissue, n=3 or 6 per group, $P < 0.01$) and at 8 weeks post-surgery in experimental protocol-3 (74 ± 40 ng/g tissue versus 8.1 ± 5.7 ng/g tissue, n=12 per group, $P < 0.01$).

Renal Sympathetic Nerve Activity measurement

A radiofrequency transmitter was used for RSNA (F50-W-F2; DSI) with nerve recording electrodes, a radiofrequency transmitter for blood pressure and heart rate (PA-C40; DSI), a receiver (RPC-3; DSI), an analog adaptor for RSNA (DL11; DSI), a digital-to-analog converter (R11CPA; DSI), and an analogue-to-digital converter (PowerLab 8/30; AD Instruments, Australia). The 24-h continuous signals of RSNA and blood pressure were sampled at 1 kHz for RSNA and at 400 Hz for blood pressure. The original RSNA signal was high-pass filtered at 50 Hz, amplified, full-wave rectified, and integrated. The RSNA, blood pressure, and heart rate data were recorded and analyzed 24 h continuously in the conscious rats. The radiotelemetry system for RSNA measurement was implanted as described previously [16, 17]. Briefly, abdominal skin and peritoneal incision were made and the transmitter was placed and secured to the abdominal wall with sutures. Subsequently, a dorsal incision was made and the accessory nerve recording electrodes

were tunneled from the abdominal to the dorsal incision subcutaneously, and then the abdominal skin was closed. The left renal artery was carefully exposed without damaging arteries, veins, lymph vessels, or nerves via the retroperitoneal approach, and renal sympathetic nerves were detected with a high-power dissecting microscope; ~2–3 mm length of the renal sympathetic nerve was carefully separated from the surrounding tissue. The electrodes were anchored to the left renal artery using 7/0 silk sutures and the electrodes were gently applied to the sympathetic nerve and embedded in a two-component silicone gel (604; Wacker-Chemie, Munich, Germany). Following hardening, the retroperitoneum and dorsal incision were closed.

Statistical analysis

All values are expressed as the mean ± SD. A p-value < 0.05 was considered statistically significant. We used SPSS software (IBM, Armonk, NY, USA) for statistical analysis. The daily average of food intake, body weight before and after the treatment, fluid intake, urine volume, UNaV, and water balance gap were analyzed by 2-tailed unpaired Student's t-test or one-way analysis of variance followed by Tukey's multiple comparison tests. The frequency distribution of blood pressure, heart rate and RSNA were analyzed by Pearson's chi-square test.

Results

We first examined the effects of HS+saline on body energy balance in SD rats. HS+saline significantly reduced body-weight gain under similar food intakes (Figure 1A and B), although HS + tap water for drinking (HS+tap) did not alter these parameters (Figure 1C and D). HS+tap exhibited a 14-fold increase in UNaV compared with NS+tap (2.9 ± 0.4 mmol/day/kg versus 39 ± 3.9 mmol/day/kg, $P < 0.01$). On the other hand, HS+saline showed 26-fold increase in UNaV compared with NS+tap (2.4 ± 0.1 mmol/day/kg versus 63 ± 7.2 mmol/day/kg , n=6 per group, $P < 0.01$). These results show that a high-salt diet combined with saline to drink induces a catabolic state in mice [6] and rats. Both HS+saline and HS+tap significantly increased fluid intake and urine volume and did not alter the water balance gap (fluid intake – urine volume), suggesting that water intake and output are not associated with the HS+saline-induced catabolic state (Supplementary figure 1A and B).

We next tested the hypothesis that HS+saline reduces heart rate and sympathetic nerve activity, and therefore reduces cardiovascular energy consumption to compensate the catabolic state. Blood pressure, heart rate, and RSNA were recorded using the radiotelemetry system in conscious rats, and their frequency distributions were analyzed to show a visual representation for the distribution of a particular variable. HS+tap marginally but significantly increased blood pressure during the active period (dark phase), but did not affect heart rate or RSNA in SD rats in the active and inactive period (light phase) (Figure 2A-D). HS+saline significantly increased blood pressure and reduced heart rate and RSNA during the active period (Figure 2A-D). Furthermore, the changes in RSNA were associated with 75% of the variability in heart rate (Figure 2E).

These data indicate that HS+saline reduces heart rate in association with the suppression of RSNA for reducing cardiovascular energy expenditure in SD rats.

A previous study reported that high salt-fed DSS rats exhibited increased renal noradrenaline content, suggesting renal sympathetic nerve activation in salt-sensitive hypertension [18]. We, therefore, next examined the effects of high salt intake on blood pressure, heart rate, and RSNA in conscious DSS rats. Treatment with either HS+tap or HS+saline significantly increased blood pressure and RSNA during the active and inactive periods in DSS rats, whereas high salt intake did not change heart rate (Figure 3A-D). The variability in heart rate, 55% was associated with changes in RSNA in DSS rats (Figure 3E). Based on these observations, we next hypothesized that DSS rats may not be able to reduce heart rate during high salt intake due to the renal sympathetic nervous activation. We next performed denervation studies.

In renal-denervated DSS rats, HS+tap and HS+saline significantly increased blood pressure, as was the case in non-denervated DSS rats (Figure 4A and B). We found that a high salt intake significantly reduced heart rate in the renal-denervated DSS rats (Figure 4A and C), suggesting that the renal sympathetic nervous system is a key mediator regulating heart rate, which may explain the insensitivity of heart rate changes to high salt intake in DSS rats. These findings also suggest that RDX can improve a catabolic state via a reduction in cardiovascular energy expenditure in high salt-fed DSS rats. Therefore, the effects of RDX on energy balance in HS+saline-treated DSS rats were examined.

HS+saline markedly increased blood pressure, UNaV, fluid intake and urine volume in the DSS rats, whereas RDX did not significantly alter the increases in these parameters (Figure 5A, B and Supplementary figure 1C). Also, the high salt-fed DSS rats

had significantly reduced body weight gain compared with the normal salt-fed DSS rats in the sham group, although there were no significant differences in food intake among all groups (Figure 5C and D). Of note, RDX significantly increased body weight gain in the high salt-fed DSS rats (Figure 5D). These findings suggest that RDX attenuates a catabolic state in high salt-fed DSS rats.

Discussion

An important finding in our study is that HS+saline reduced RSNA, which was coupled with a reduction in heart rate, in conscious SD rats. In our earlier mouse study, we showed that the same high-salt model induced a reduction in heart rate in conscious C57BL/6J mice [6]. In contrast, we did not observe similar cardiovascular or renal sympathetic nerve responses to high salt intake in conscious DSS rats. Alternatively, the DSS rats exhibited increased RSNA during high salt intake, suggesting that salt-sensitive animals are not able to reduce heart rate due to the increased renal sympathetic nervous activation. Renal-denervated DSS rats exhibited a reduction in heart rate during high salt intake. These results suggest that a high-salt intake reduces heart rate via the suppression of RSNA in a healthy subject (Supplementary figure 2A). Possibly, the insensitivity of the heart to changes in heart rate following high salt intake due to the renal sympathetic nervous activation may be an additional critical diagnostic factor for salt-sensitive hypertension (Supplementary figure 2B). One of the limitations of this study is that we could not clarify why DSS rats significantly increased RSNA but not heart rate during high salt intake.

The sympathetic nervous system is one of the master mediators of cardiovascular function, which regulates various unconscious cardiovascular actions, including vascular tone and heartbeat to maintain hemodynamics homeostasis [19]. The renal sympathetic nervous system has both efferent and afferent nerve activity; therefore, it can exert an influence on the sympathetic nerve regulation of central including baroreflex [20] and other organs [21, 22]. Foss et al. [21] reported that renal afferent sympathetic nerve denervation suppressed the bradykinin-induced increase in heart rate in rats, suggesting that the renal sympathetic nervous system has afferent nerve activity and that it influences heartbeat. In fact, RDX did not significantly alter UNaV in high salt-fed DSS rats,

suggesting that RDX-induced decreases in heart rate were not mediated by the efferent renal nervous system, which regulates sodium reabsorption in the kidney [23, 24]. Therefore, RDX may decrease heart rate via the deletion of abnormally activated renal afferent nerves in high salt-fed DSS rats. However, we did not measure sodium intake and UNaV for several days before and after RDX. Thus, we might not be able to evaluate the effects of RDX on urinary sodium excretion precisely. In addition, the RDX approach used in the present study cut both efferent and afferent renal sympathetic nervous system. The roles of renal efferent and/or afferent nerve activity in the regulation of heartbeat remain to be fully elucidated. Previous studies also found that the renal efferent nerve activity regulates kidney-driven humoral factors such as renin, which ultimately produces angiotensin II in the circulatory system [23, 25]. This state-of-affairs could influence heart rate. Thus, we cannot rule out a possibility that RDX alters certain renal humoral factors via the deletion of renal efferent sympathetic nerve activity, thereby reducing heart rate, independent of renal afferent sympathetic nerve activity.

Heartbeat is an essential life activity and heart rate is strongly associated with the average life-span of the majority of animals [7]. Previous studies have also reported that heart rate is negatively associated with longevity in healthy humans and patients with cardiovascular diseases, and that a high heart rate is a risk factor of cardiovascular events, suggesting that changes in heart rate are associated with the protection of the cardiovascular system [7, 8]. Therefore, the reduction in heart rate on a high salt diet could provide an important cardiovascular protective factor and RDX may prevent cardiovascular events via decreasing heart rate in salt-sensitive hypertension. In contrast, the cardiovascular system, in particular, pumping of the heart, is an important site of basal metabolism and energy consumption in the body, which can contribute to energy

metabolism in the body [7]. A previous study reported that an elevated heart rate leads to an increase in oxygen consumption in mice, indicating enhanced energy expenditure [14]. Therefore, changes in heart rate during high salt intake are important from the perspective of not only the cardiovascular function, but also energy metabolism. In the present study, we showed that renal-denervated DSS rats exhibited an attenuated catabolic state on a high-salt diet, suggesting that RDX can improve total body energy balance, at least partly via a reduction in heart rate and cardiovascular energy expenditure. This improved body energy state also contributes to cardiovascular protection as a catabolic state, including muscle wasting and cachexia, is an exacerbating factor for chronic heart failure [9, 10]. In addition, RDX can also alter energy metabolism in other organs, including the kidney, liver, and muscle, which may possibly contribute to the attenuation of the salt-driven catabolic state [13, 26-28]

In our previous long-term salt and water balance study in humans, we found that dietary salt was excreted into the urine by a renal concentrating mechanism, which was associated with a reduction in water intake and increases in glucocorticoids, catabolic hormones, levels [3]. These findings suggest that the renal Na^+ concentration process during high salt intake induces a catabolic state and increases endogenous metabolic water production in humans [3]. However, there are major differences regarding the salt-driven catabolism between humans and rodents [3, 6]. Rodents have a stronger renal concentration ability than humans, which enables the kidneys to concentrate dietary salt into the urine easily without inducing catabolism [6]. In addition, mice and rats exhibit markedly increased water intake under a high salt diet, indicating that a high salt diet alone cannot mimic the salt and water metabolism in humans [3]. HS+tap treatment did not induce a catabolic state in mice [6] or rats. Therefore, high salt feeding with saline to

drink, extreme high salt intake and/or high salt diet without free water, is required to observe high salt-induced catabolism in rodents [6]. Moreover, saline to drink could affect behavior such as locomotor activity in rodents, which may alter energy expenditure and induce a catabolic state in the HS+saline model. The effects of saline for drinking on behavior should be addressed in future studies. This HS+saline model is one of the limitations in animal studies involving rodents and thus a novel animal model requiring lower salt levels may be required to mimic the salt and water metabolism in humans.

In the previous study, we found that HS+saline significantly increased daily food intake in mice [6]. Interestingly, we observed that a 12 g/day high-salt diet led our human subjects to complain about being hungry all the time [3]. We concluded that a high-salt intake increases appetite to compensate for the salt-driven catabolic state [3, 6]. However, HS+saline, irrespective of RDX, did not increase food intake in the SD or DSS rats. Further investigations are required to explain the difference in high salt-stimulated appetite between humans, mice, and rats, although there may be differences between *Mus musculus* and *Rattus norvegicus* in that regard.

The renal sympathetic nervous system activation increases blood pressure and thus RDX is an attractive therapeutic tool for the treatment of hypertensive patients [29, 30]. In the present study, we found that high salt intake enhanced RSNA and raised blood pressure in DSS rats. However, the effects of RDX on blood pressure in salt-sensitive hypertension were not apparent, as has been reported in other studies [31-33]. These findings suggest that abnormal renal sympathetic nervous activation is not a dominant cause of salt-sensitive hypertension and that RDX reduced heart rate and attenuated a catabolic state in high salt-fed DSS rats independent of blood pressure-related effects.

One limitation of this study is that we measured the long-term effect of RDX on blood pressure only using a tail-cuff method.

The effects of RDX on blood pressure in resistant hypertensive patients have been controversial in clinical trials [34-36]. However, improvements in the equipment used, improved nerve ablation techniques, adherence to anti-hypertensive drugs, and clinical study settings have led to significant decreases in the blood pressure of patients with resistant hypertension [29, 30]. There is growing evidence for the beneficial effects of RDX on resistant hypertension in clinical studies, and the renal sympathetic nerve ablation technic would become a novel treatment for hypertension in the future. On the other hand, clinical studies have shown the beneficial effects of RDX beyond its blood pressure-lowering properties. Pokushalov et al. [37] reported that the combination therapy of pulmonary vein isolation (PVI) with RDX significantly decreased blood pressure and the recurrence of arterial fibrillation (AF), compared to PVI therapy alone in patients with refractory symptomatic AF and resistant hypertension. Feyz et al. [38] reported that combination therapy with PVI and RDX significantly reduced total AF time one year following surgery. Ambulatory blood pressure monitoring in this study revealed that RDX did not alter blood pressure in the same patients, a finding indicating that RDX could improve AF independently of blood pressure. In addition, RDX led to blood pressure-independent improvements of apnea/hypoxia indices, emotional reactions, and sleep disorders in patients with sleep apnea syndrome [39, 40]. In terms of cardiovascular markers, a previous meta-analysis revealed that RDX improved left ventricular mass index, central augmentation index, and carotid-femoral pulse wave velocity independently of blood pressure [41]. In animal studies, Watanabe et al. [42] and Jiang et al. [43] reported the blood pressure-independent cardioprotective effects of RDX in DSS

and spontaneously hypertensive rats. These basic and clinical findings suggest that RDX can also decrease the risk of cardiovascular disease through methods other than a reduction in blood pressure. In the present study, the RDX-induced changes in cardiovascular energy expenditure and body energy state may partially account for the blood pressure-independent beneficial effects of RDX on the cardiovascular system. In fact, the SPYRAL HTN-OFF MED trial recently revealed that catheter-based renal denervation reduces heart rate in hypertensive patients who were not receiving anti-hypertensive medications [44]. This observation suggests that renal denervation could also decrease heartbeat in humans [44]. Salt intake was not manipulated and presumably remained constant in that study. The re-evaluation of the beneficial RDX effects on heart rate and cardiovascular energy metabolism, in addition to blood pressure, is required in clinical studies. Also, salt intake and salt-sensitivity should be considered in future trials.

In summary, we found that the renal sympathetic nervous system is one of the major regulators of a cardiac beat during high salt intake (Supplementary figure 2) and that renal denervation could attenuate the salt-driven catabolic state at least partly via a reduction in cardiovascular energy expenditure in salt-sensitive hypertension. We would need to evaluate the blood pressure-independent beneficial effects of renal denervation on cardiovascular energy metabolism.

Conflicts of interests:

The authors have nothing to disclose.

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Author contributions:

NM, KK, YF, DY, LL, YZ and TM performed animal experiments. KK, TM, YK, TY, JT and AN provided essential material and contributed to the design of the experiments. SK measured, analyzed and interpreted tissue noradrenaline content data. NM and YF performed animal surgery and the radiotelemetry measurements. NM, KK and YF analyzed the radiotelemetry data. NM, KK, DN, DY, JT and AN designed and planned the experiments and analyzed and interpreted data. NM, KK, DN, FL, JT and AN wrote the manuscript. YK, TY, FL, JT and AN supervised the research project.

References

1. Bonventre JV, Leaf A. Sodium homeostasis: steady states without a set point. *Kidney Int.* 1982;21:880-883.

2. Hollenberg NK. Set point for sodium homeostasis: surfeit, deficit, and their implications. *Kidney Int.* 1980;17:423-429.

3. Rakova N, Kitada K, Lerchl K, Dahlmann A, Birukov A, Daub S, et al. Increased salt consumption induces body water conservation and decreases fluid intake. *J Clin Invest.* 2017;127:1932-1943.

4. Lerchl K, Rakova N, Dahlmann A, Rauh M, Goller U, Basner M, et al. Agreement between 24-hour salt ingestion and sodium excretion in a controlled environment. *Hypertension.* 2015;66:850-857.

5. Rakova N, Juttner K, Dahlmann A, Schroder A, Linz P, Kopp C, et al. Long-term space flight simulation reveals infradian rhythmicity in human Na(+) balance. *Cell Metab.* 2013;17:125-131.

6. Kitada K, Daub S, Zhang Y, Klein JD, Nakano D, Pedchenko T, et al. High salt intake reprioritizes osmolyte and energy metabolism for body fluid conservation. *J Clin Invest.* 2017;127:1944-1959.

7. Boudoulas KD, Borer JS, Boudoulas H. Heart Rate, Life Expectancy and the Cardiovascular System: Therapeutic Considerations. *Cardiology.* 2015;132:199-212.

8. Palatini P. Role of elevated heart rate in the development of cardiovascular disease in hypertension. *Hypertension.* 2011;58:745-750.

9. Araujo JP, Lourenco P, Rocha-Goncalves F, Ferreira A, Bettencourt P. Nutritional markers and prognosis in cardiac cachexia. *Int J Cardiol.* 2011;146:359-363.

10. Loncar G, Fulster S, von Haehling S, Popovic V. Metabolism and the heart: an overview of muscle, fat, and bone metabolism in heart failure. *Int J Cardiol.* 2013;162:77-85.

11. de Lucia C, Piedepalumbo M, Paolisso G, Koch WJ. Sympathetic nervous system in age-related cardiovascular dysfunction: Pathophysiology and therapeutic perspective. *Int J Biochem Cell Biol.* 2019;108:29-33.

12. Sufiun A, Rafiq K, Fujisawa Y, Rahman A, Mori H, Nakano D, et al. Effect of dipeptidyl peptidase-4 inhibition on circadian blood pressure during the development of salt-dependent hypertension in rats. *Hypertens Res.* 2015;38:237-243.

13. Rafiq K, Fujisawa Y, Sherajee SJ, Rahman A, Sufiun A, Kobori H, et al. Role of the renal sympathetic nerve in renal glucose metabolism during the development of type 2 diabetes in rats. *Diabetologia.* 2015;58:2885-2898.

14. Lei B, Nakano D, Fujisawa Y, Liu Y, Hitomi H, Kobori H, et al. N-type calcium channel inhibition with cilnidipine elicits glomerular podocyte protection independent of sympathetic nerve inhibition. *J Pharmacol Sci.* 2012;119:359-367.

15. Luippold G, Beilharz M, Muhlbauer B. Chronic renal denervation prevents glomerular hyperfiltration in diabetic rats. *Nephrol Dial Transplant.* 2004;19:342-347.

16. Salman IM, Sarma Kandukuri D, Harrison JL, Hildreth CM, Phillips JK. Direct conscious telemetry recordings demonstrate increased renal sympathetic nerve activity in rats with chronic kidney disease. *Front Physiol.* 2015;6:218.

17. Stocker SD, Muntzel MS. Recording sympathetic nerve activity chronically in rats: surgery techniques, assessment of nerve activity, and quantification. *Am J*

Physiol Heart Circ Physiol. 2013;305:H1407-1416.

18. Nagasu H, Satoh M, Fujimoto S, Tomita N, Sasaki T, Kashihara N. Azelnidipine attenuates glomerular damage in Dahl salt-sensitive rats by suppressing sympathetic nerve activity. *Hypertens Res.* 2012;35:348-355.

19. Cohn JN. Sympathetic nervous system activity and the heart. *Am J Hypertens.* 1989;2:353S-356S.

20. Thames MD, Miller BD, Abboud FM. Baroreflex regulation of renal nerve activity during volume expansion. *Am J Physiol.* 1982;243:H810-814.

21. Foss JD, Wainford RD, Engeland WC, Fink GD, Osborn JW. A novel method of selective ablation of afferent renal nerves by periaxonal application of capsaicin. *Am J Physiol Regul Integr Comp Physiol.* 2015;308:R112-122.

22. Schlaich MP, Esler MD, Fink GD, Osborn JW, Euler DE. Targeting the sympathetic nervous system: critical issues in patient selection, efficacy, and safety of renal denervation. *Hypertension.* 2014;63:426-432.

23. Prosnitz EH, DiBona GF. Effect of decreased renal sympathetic nerve activity on renal tubular sodium reabsorption. *Am J Physiol.* 1978;235:F557-563.

24. DiBona GF. Neural regulation of renal tubular sodium reabsorption and renin secretion. *Fed Proc.* 1985;44:2816-2822.

25. DiBona GF, Kopp UC. Neural control of renal function. *Physiol Rev.* 1997;77:75-197.

26. Rafiq K, Noma T, Fujisawa Y, Ishihara Y, Arai Y, Nabi AH, et al. Renal sympathetic denervation suppresses de novo podocyte injury and albuminuria in rats with aortic regurgitation. *Circulation.* 2012;125:1402-1413.

27. Chen W, Chang Y, He L, Jian X, Li L, Gao L, et al. Effect of renal sympathetic

denervation on hepatic glucose metabolism and blood pressure in a rat model of insulin resistance. *J Hypertens.* 2016;34:2465-2474.

28. Tsioufis C, Dimitriadis K, Kasiakogias A, Kalos T, Liatakis I, Koutra E, et al. Effects of multielectrode renal denervation on elevated sympathetic nerve activity and insulin resistance in metabolic syndrome. *J Hypertens.* 2017;35:1100-1108.

29. Kandzari DE, Bohm M, Mahfoud F, Townsend RR, Weber MA, Pocock S, et al. Effect of renal denervation on blood pressure in the presence of antihypertensive drugs: 6-month efficacy and safety results from the SPYRAL HTN-ON MED proof-of-concept randomised trial. *Lancet.* 2018;391:2346-2355.

30. Azizi M, Schmieder RE, Mahfoud F, Weber MA, Daemen J, Davies J, et al. Endovascular ultrasound renal denervation to treat hypertension (RADIANCE-HTN SOLO): a multicentre, international, single-blind, randomised, sham-controlled trial. *Lancet.* 2018;391:2335-2345.

31. Foss JD, Fiege J, Shimizu Y, Collister JP, Mayerhofer T, Wood L, et al. Role of afferent and efferent renal nerves in the development of AngII-salt hypertension in rats. *Physiol Rep.* 2018;6.

32. Nagasu H, Satoh M, Kuwabara A, Yorimitsu D, Sakuta T, Tomita N, et al. Renal denervation reduces glomerular injury by suppressing NAD(P)H oxidase activity in Dahl salt-sensitive rats. *Nephrol Dial Transplant.* 2010;25:2889-2898.

33. Tudorancea I, Lohmeier TE, Alexander BT, Pieptu D, Serban DN, Iliescu R. Reduced Renal Mass, Salt-Sensitive Hypertension Is Resistant to Renal Denervation. *Front Physiol.* 2018;9.

34. Symplicity HTNI, Esler MD, Krum H, Sobotka PA, Schlaich MP, Schmieder RE, et al. Renal sympathetic denervation in patients with treatment-resistant

hypertension (The Symplicity HTN-2 Trial): a randomised controlled trial. *Lancet.* 2010;376:1903-1909.

35. Bhatt DL, Kandzari DE, O'Neill WW, D'Agostino R, Flack JM, Katzen BT, et al. A controlled trial of renal denervation for resistant hypertension. *N Engl J Med.* 2014;370:1393-1401.

36. Kario K, Ogawa H, Okumura K, Okura T, Saito S, Ueno T, et al. SYMPLICITY HTN-Japan - First Randomized Controlled Trial of Catheter-Based Renal Denervation in Asian Patients. *Circ J.* 2015;79:1222-1229.

37. Pokushalov E, Romanov A, Corbucci G, Artyomenko S, Baranova V, Turov A, et al. A randomized comparison of pulmonary vein isolation with versus without concomitant renal artery denervation in patients with refractory symptomatic atrial fibrillation and resistant hypertension. *J Am Coll Cardiol.* 2012;60:1163-1170.

38. Feyz L, Theuns DA, Bhagwandien R, Strachinaru M, Kardys I, Van Mieghem NM, et al. Atrial fibrillation reduction by renal sympathetic denervation: 12 months' results of the AFFORD study. *Clin Res Cardiol.* 2018;10:018-1391.

39. Warchol-Celinska E, Prejbisz A, Kadziela J, Florczak E, Januszewicz M, Michalowska I, et al. Renal Denervation in Resistant Hypertension and Obstructive Sleep Apnea: Randomized Proof-of-Concept Phase II Trial. *Hypertension.* 2018;72:381-390.

40. Krawczyk-Ozog A, Tokarek T, Moczala K, Siudak Z, Dziewierz A, Mielecki W, et al. Long-term quality of life and clinical outcomes in patients with resistant hypertension treated with renal denervation. *Postepy Kardiol Interwencyjnej.* 2016;12:329-333.

41. Kordalis A, Tsiachris D, Pietri P, Tsioufis C, Stefanadis C. Regression of organ damage following renal denervation in resistant hypertension: a meta-analysis. *J Hypertens.* 2018;36:1614-1621.

42. Watanabe H, Iwanaga Y, Miyaji Y, Yamamoto H, Miyazaki S. Renal denervation mitigates cardiac remodeling and renal damage in Dahl rats: a comparison with beta-receptor blockade. *Hypertens Res.* 2016;39:217-226.

43. Jiang W, Tan L, Guo Y, Li X, Tang X, Yang K. Effect of renal denervation procedure on left ventricular hypertrophy of hypertensive rats and its mechanisms. *Acta Cir Bras.* 2012;27:815-820.

44. Bohm M, Mahfoud F, Townsend RR, Kandzari DE, Pocock S, Ukena C, et al. Ambulatory heart rate reduction after catheter-based renal denervation in hypertensive patients not receiving anti-hypertensive medications: data from SPYRAL HTN-OFF MED, a randomized, sham-controlled, proof-of-concept trial. *Eur Heart J.* 2019;40:743-751.

Figure legends

Figure 1. Effects of high salt intake on food intake and body weight gain in Sprague Dawley rats.

(A) Daily food intake (average) in Sprague-Dawley (SD) rats on a 0.2% NaCl diet with tap water to drink (NS+tap) or a 4% NaCl diet with saline to drink (HS+saline) (n=6 per group). (B) Body weight before and 6 weeks after the special diets in the same NS+tap or HS+saline-fed SD rats. (C) Daily food intake (average) in SD rats on NS+tap or a 4% NaCl diet with tap water to drink (HS+tap) (n=6 or 7 per group). (D) Body weight before and 6 weeks after the special diets in the same NS+tap or HS+tap SD rats. All values are expressed as the mean ± SD.

Figure 2. Effects of high salt intake on blood pressure, heart rate and renal sympathetic nerve activity in conscious Sprague Dawley rats.

(A) Renal sympathetic nerve activity (RSNA), heart rate and mean arterial pressure in conscious Sprague-Dawley (SD) rats (n=3) on a 0.2% NaCl diet with tap water to drink (NS tap), a 4% NaCl diet with tap water to drink (HS tap) or a 4% NaCl diet with saline to drink (HS saline) over a 12-day period. (B) Mean arterial pressure distribution in the same SD rats during each salt intake phase. Data were analyzed in a dark phase (active period) and a light phase (inactive period) separately. (C) Heart rate distribution in the same SD rats during each salt intake phase. (D) RSNA distribution in the same SD rats during each salt intake phase. (E) Relationship between heart rate (bpm) and RSNA (μV・s) for all hourly average data (n=288) in the same SD rats presented in panel A. All values are expressed as the mean ± SD.

Figure 3. Effects of high salt intake on blood pressure, heart rate and renal sympathetic nerve activity in conscious Dahl salt-sensitive rats.

(A) Renal sympathetic nerve activity (RSNA), heart rate and mean arterial pressure in conscious Dahl salt-sensitive (DSS) rats (n=3) on a 0.2% NaCl diet with tap water to drink (NS tap), a 4% NaCl diet with tap water to drink (HS tap) or a 4% NaCl diet with saline to drink (HS saline) over a 12-day period. (B) Mean arterial pressure distribution in the same DSS rats during each salt intake phase. Data were analyzed in a dark phase (active period) and a light phase (inactive period) separately. (C) Heart rate distribution in the same DSS rats during each salt intake phase. (D) RSNA distribution in the same DSS rats during each salt intake phase. (E) Relationship between heart rate (bpm) and RSNA (μV・s) for all hourly average data (n=288) in the same DSS rats presented in panel A. All values are expressed as the mean ± SD.

Figure 4. Effects of high salt intake on blood pressure and heart rate in conscious renal-denervated Dahl salt-sensitive rats.

(A) Heart rate and mean arterial pressure in conscious renal-denervated Dahl salt-sensitive (DSS) rats (n=3) on a 0.2% NaCl diet with tap water to drink (NS tap), a 4% NaCl diet with tap water to drink (HS tap) or a 4% NaCl diet with saline to drink (HS saline) over a 12-day period. (B) Mean arterial pressure distribution in the same DSS rats during each salt intake phase. Data were analyzed in a dark phase (active period) and a light phase (inactive period) separately. (C) Heart rate distribution in the same DSS rats during each salt intake phase. All values are expressed as the mean ± SD.

Figure 5. Effects of renal denervation on blood pressure, food intake and body

weight gain in high salt-fed Dahl salt-sensitive rats.

(A) Systolic blood pressure (SBP) over a 6-week period in sham-operated Dahl salt-sensitive (DSS) rats on a 0.2% NaCl diet with tap water to drink (sham+NS+tap) or a 4% NaCl diet with saline to drink (sham+HS+saline) and renal-denervated DSS rats on a 4% NaCl diet with saline to drink (RDX+HS+saline) (n=6 per group). (B) Urinary sodium excretion (UNaV) in the same DSS rats (n=6 per group). (C) Daily food intake (average) in the same DSS rats. (D) Body weight over a 42-day period in the same DSS rats. All values are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, versus sham+NS+tap, $^{\dagger}P < 0.05$, versus sham+HS+saline.

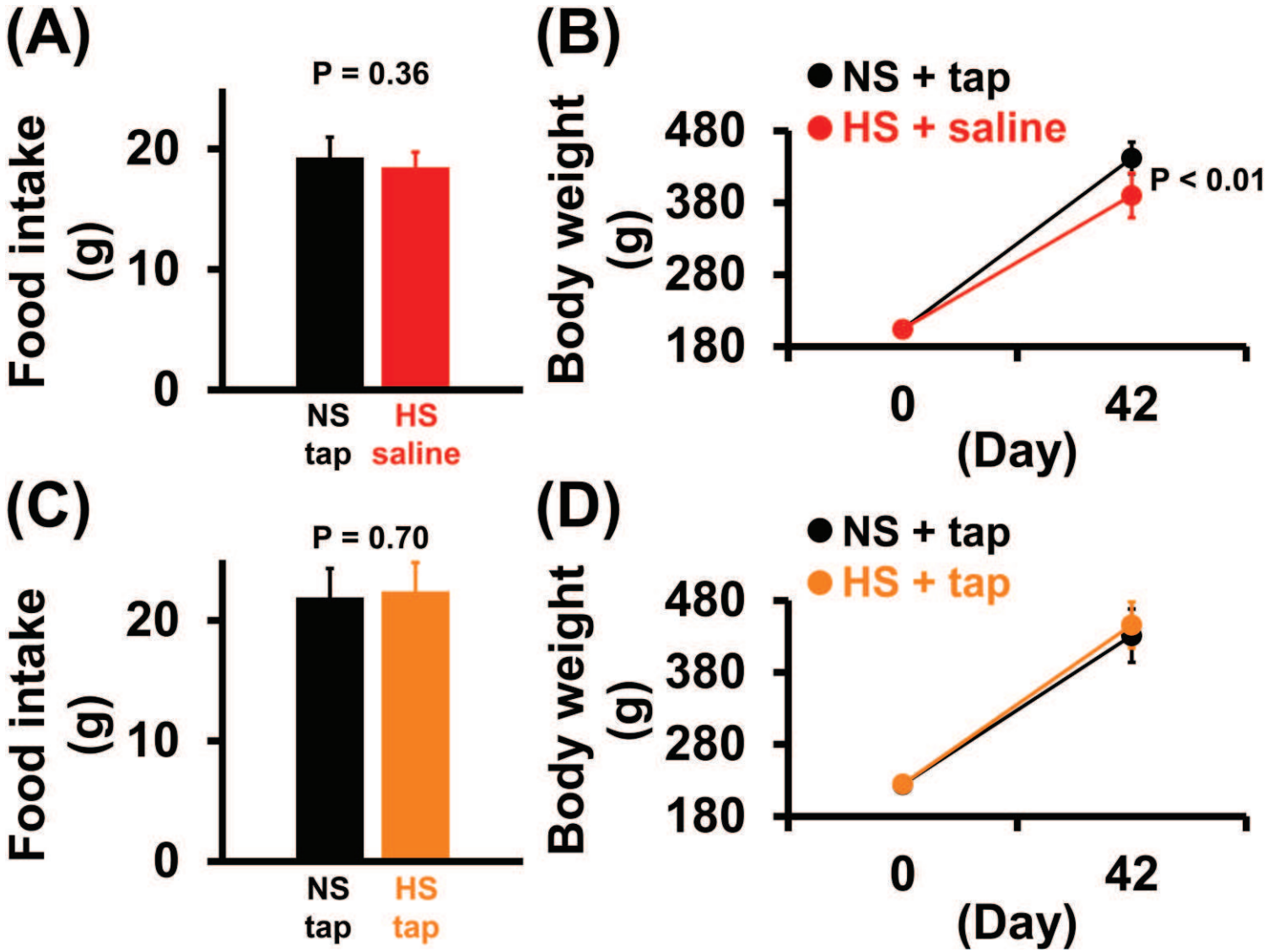

(A)
P = 0.36
Food intake (g)
20
10
0
NS tap
HS saline
(B)
NS + tap
HS + saline
Body weight (g)
480
380
280
180
P < 0.01
0
42
(Day)
(C)
P = 0.70
Food intake (g)
20
10
0
NS tap
HS tap
(D)
NS + tap
HS + tap
Body weight (g)
480
380
280
180
0
42
(Day)

Figure 1

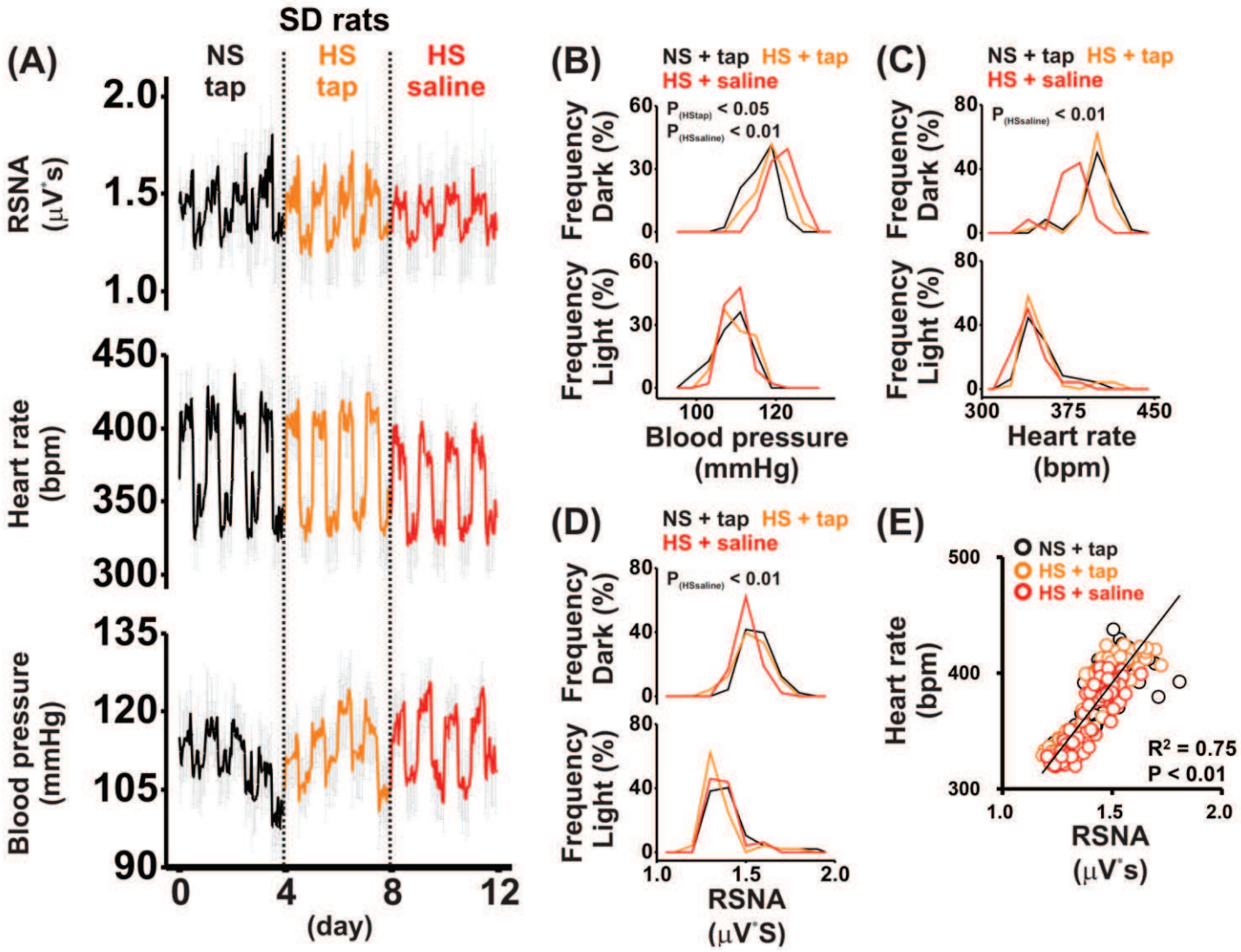

SD rats
(A)
NS tap
HS tap
HS saline
RSNA (μV*s)
2.0
1.5
1.0
Heart rate (bpm)
450
400
350
300
Blood pressure (mmHg)
135
120
105
90
0
4
8
12
(day)
(B)
NS + tap
HS + tap
HS + saline
P(HStap) < 0.05
P(HSsaline) < 0.01
Frequency Dark (%)
Frequency Light (%)
60
30
0
100
120
Blood pressure (mmHg)
(C)
NS + tap
HS + tap
HS + saline
P(HSsaline) < 0.01
Frequency Dark (%)
Frequency Light (%)
80
40
0
300
375
450
Heart rate (bpm)
(D)
NS + tap
HS + tap
HS + saline
P(HSsaline) < 0.01
Frequency Dark (%)
Frequency Light (%)
80
40
0
1.0
1.5
2.0
RSNA (μV*S)
(E)
NS + tap
HS + tap
HS + saline
Heart rate (bpm)
500
400
300
R² = 0.75
P < 0.01
1.0
1.5
2.0
RSNA (μV*s)

Figure 2

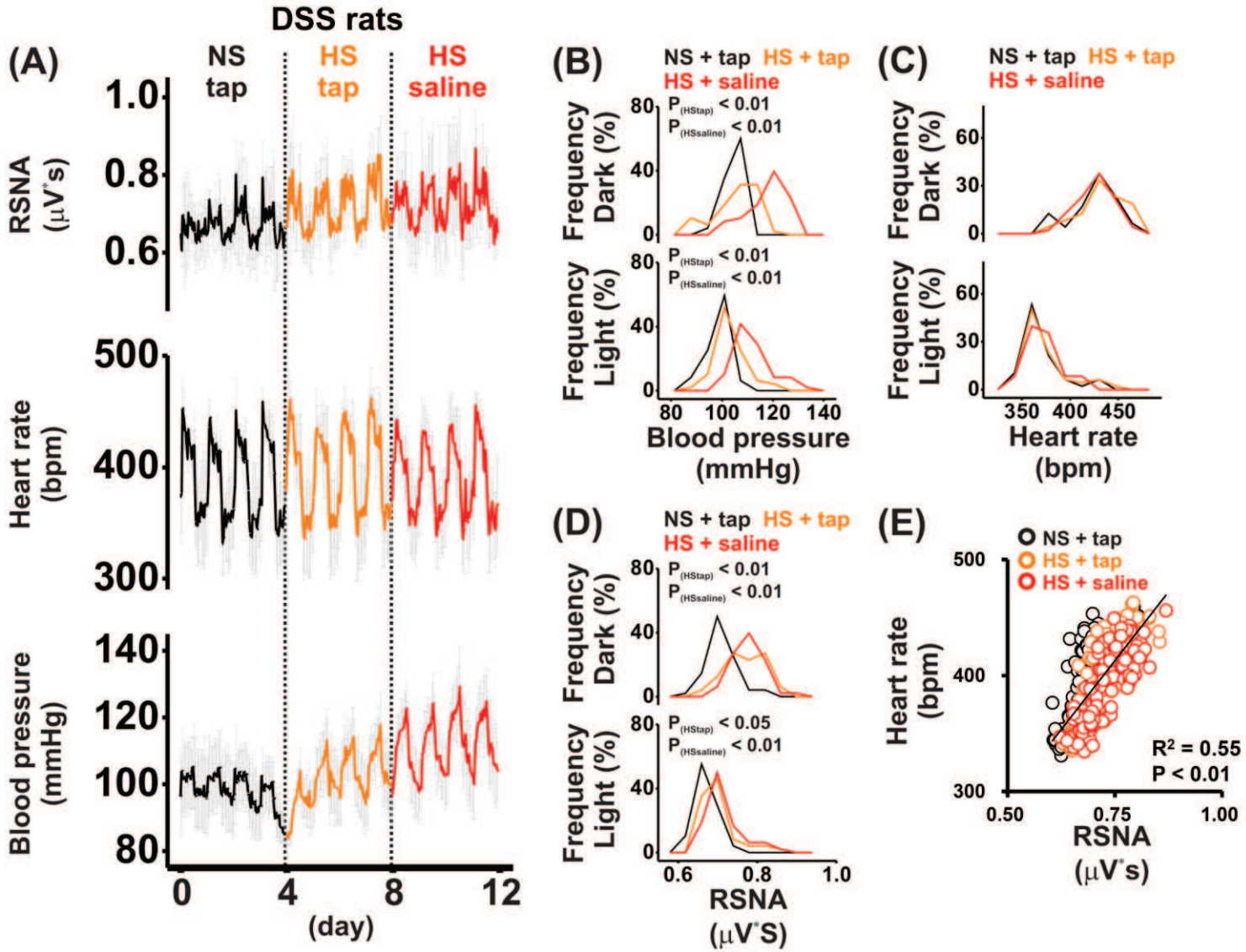

DSS rats
(A)
NS tap
HS tap
HS saline
RSNA (μV*s)
1.0
0.8
0.6
Heart rate (bpm)
500
400
300
Blood pressure (mmHg)
140
120
100
80
0
4
8
12
(day)
(B)
NS + tap
HS + tap
HS + saline
P(HStap) < 0.01
P(HSsaline) < 0.01
Frequency Dark (%)
Frequency Light (%)
80
40
0
80
100
120
140
Blood pressure (mmHg)
(C)
NS + tap
HS + tap
HS + saline
Frequency Dark (%)
Frequency Light (%)
60
30
0
350
400
450
Heart rate (bpm)
(D)
NS + tap
HS + tap
HS + saline
P(HStap) < 0.01
P(HSsaline) < 0.01
P(HStap) < 0.05
P(HSsaline) < 0.01
Frequency Dark (%)
Frequency Light (%)
0.6
0.8
1.0
RSNA (μV*S)
(E)
NS + tap
HS + tap
HS + saline
Heart rate (bpm)
500
400
300
R² = 0.55
P < 0.01
0.50
0.75
1.00
RSNA (μV*s)

Figure 3

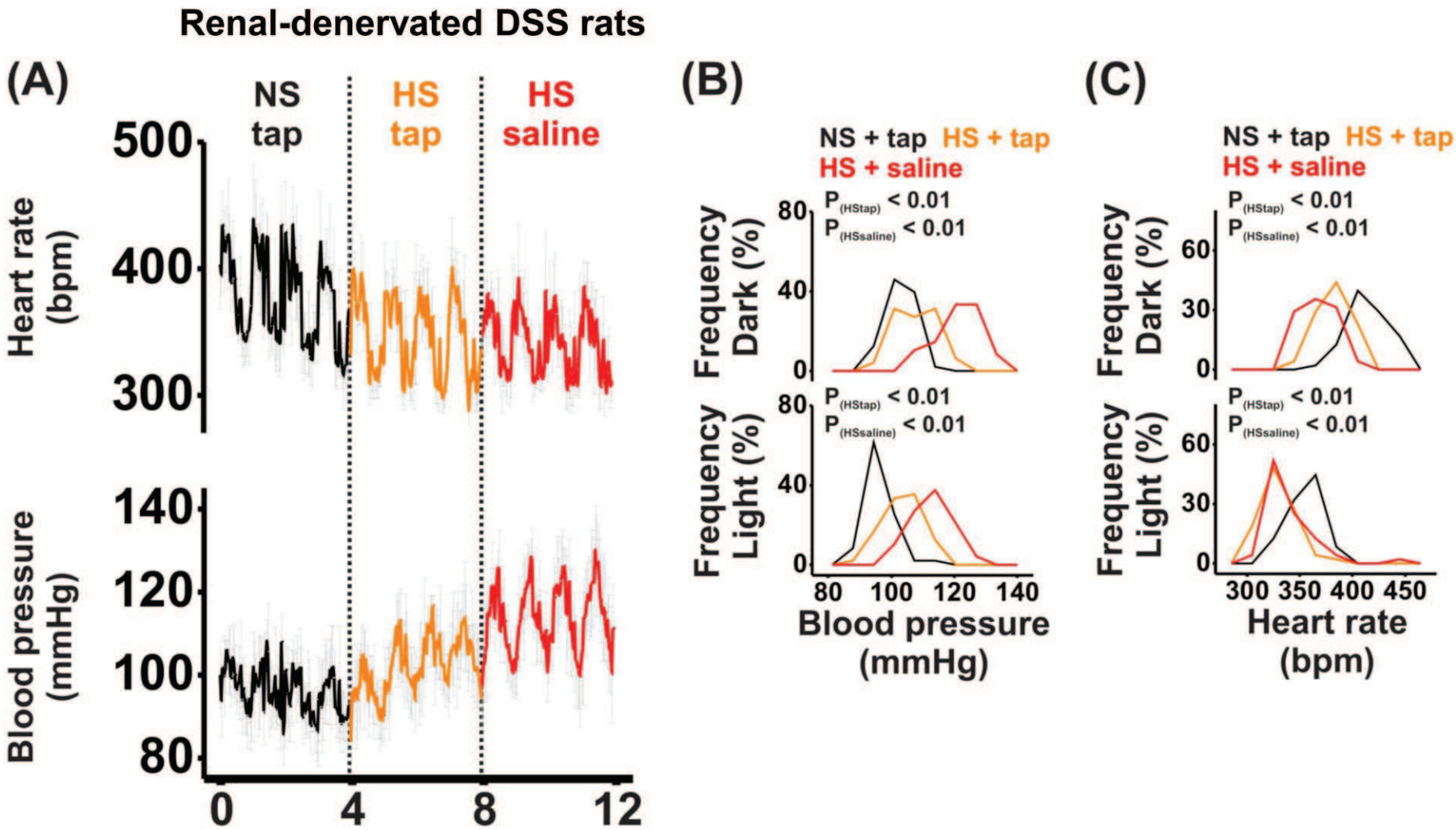

Renal-denervated DSS rats
(A)
NS tap
HS tap
HS saline
Heart rate (bpm)
500
400
300
Blood pressure (mmHg)
140
120
100
80
0
4
8
12
(B)
NS + tap
HS + tap
HS + saline
P(HStap) < 0.01
P(HSsaline) < 0.01
Frequency Dark (%)
Frequency Light (%)
80
40
0
80
100
120
140
Blood pressure (mmHg)
(C)
NS + tap
HS + tap
HS + saline
P(HStap) < 0.01
P(HSsaline) < 0.01
Frequency Dark (%)
Frequency Light (%)
60
30
0
300
350
400
450
Heart rate (bpm)

Figure 4

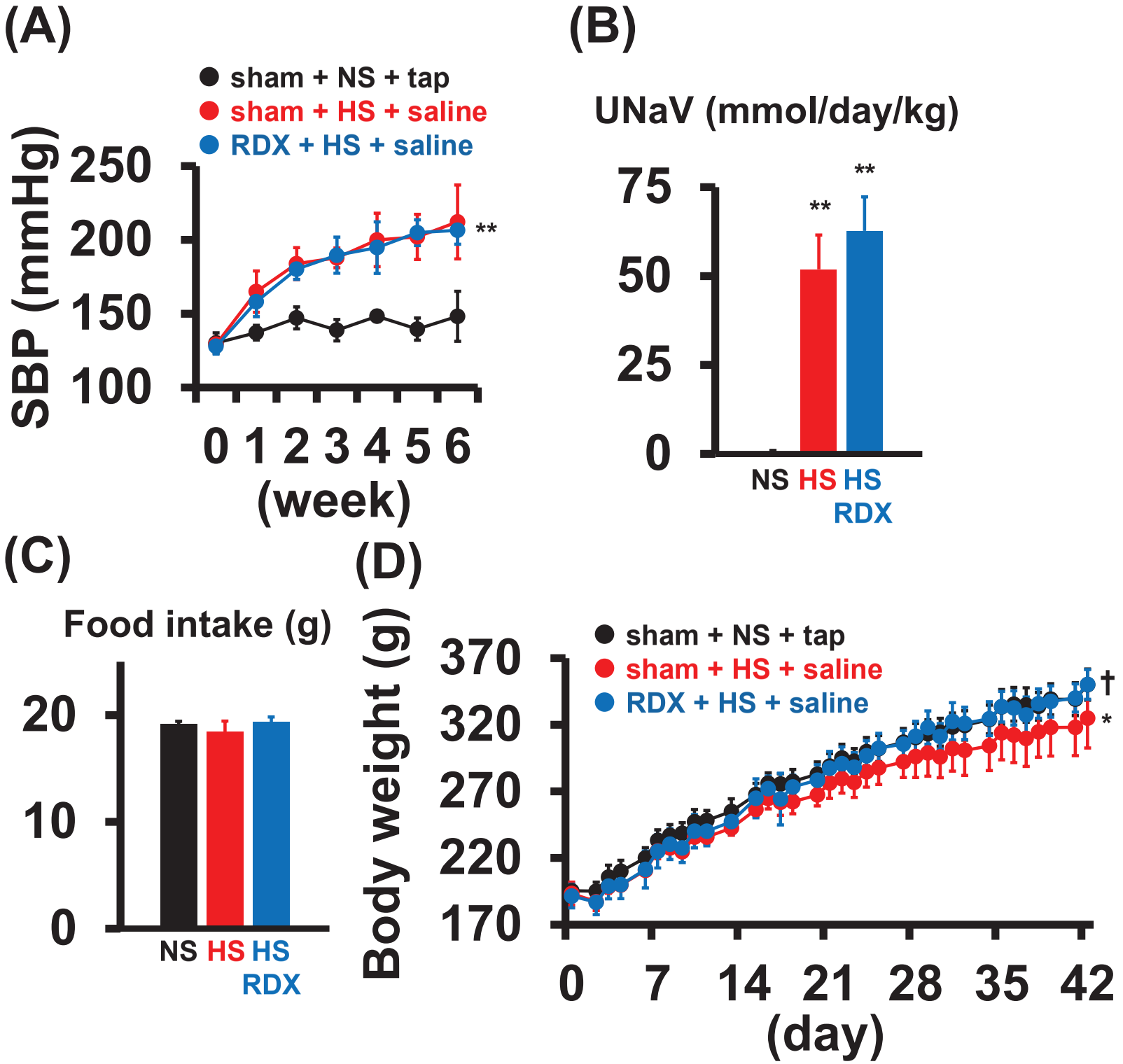
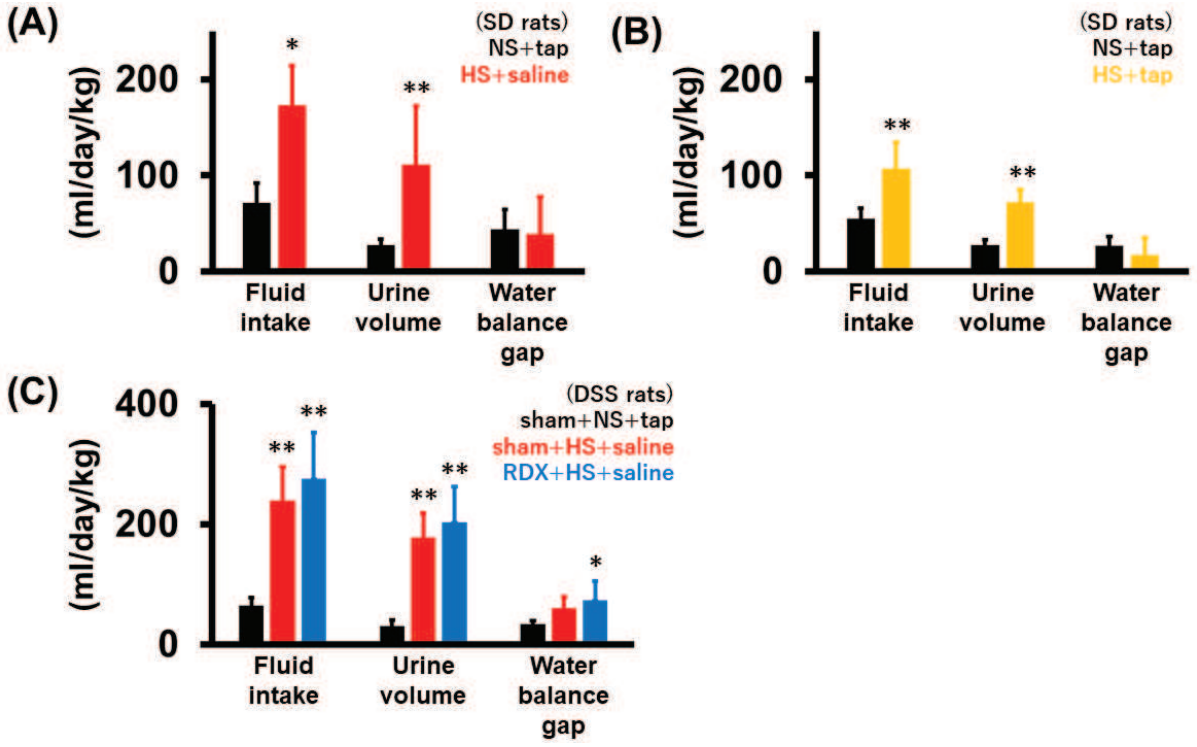
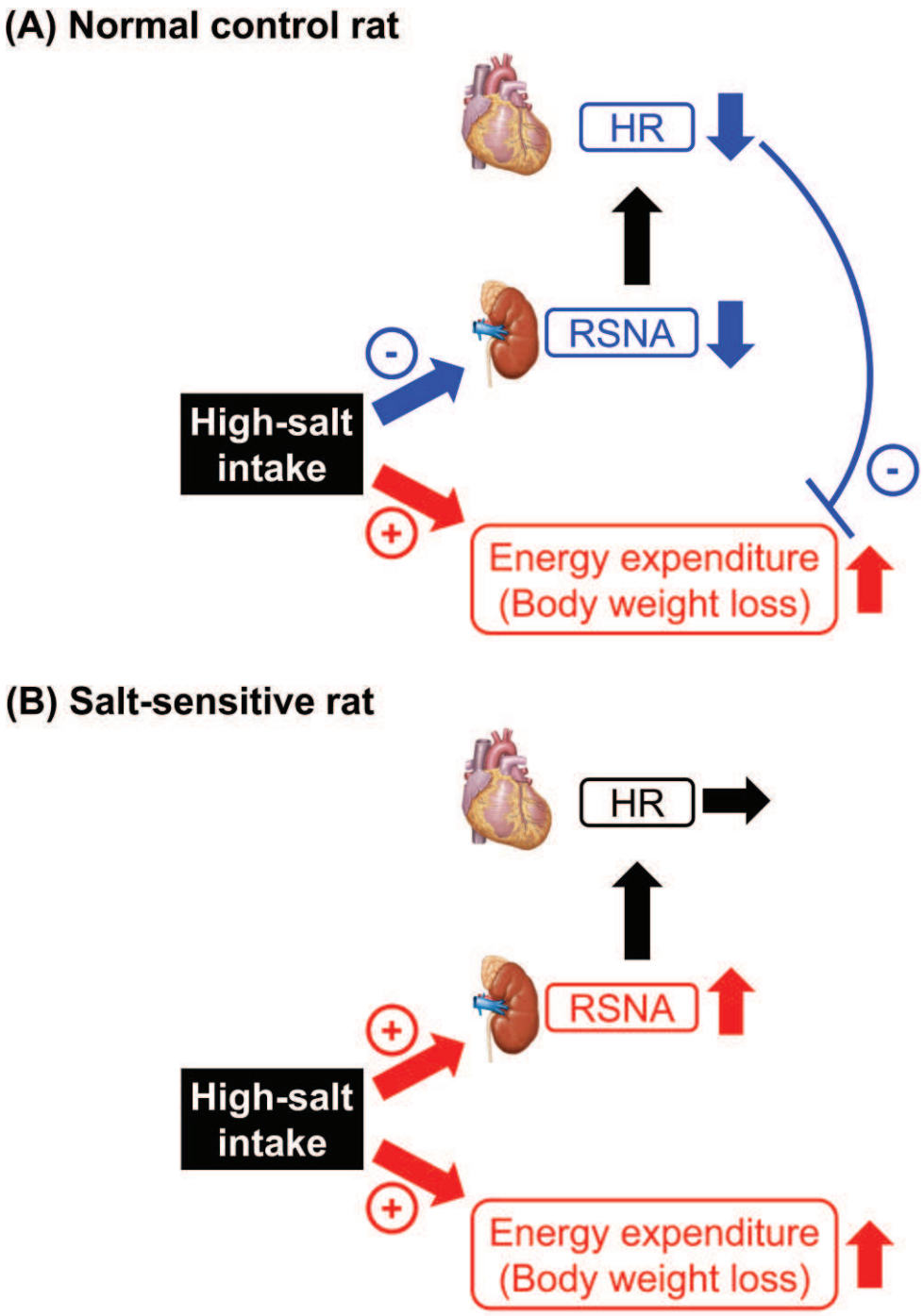

(A)
sham + NS + tap
sham + HS + saline
RDX + HS + saline
SBP (mmHg)
250
200
150
100
**
0 1 2 3 4 5 6
(week)
(B)
UNaV (mmol/day/kg)
75
50
25
0
**
**
NS HS HS
RDX
(C)
Food intake (g)
20
10
0
NS HS HS
RDX
(D)
Body weight (g)
370
320
270
220
170
sham + NS + tap
sham + HS + saline
RDX + HS + saline
†
*
0 7 14 21 28 35 42
(day)

Figure 5



Supplementary figure 1. The effect of high salt intake on fluid intake, urine volume and water balance gap.

(A) Fluid intake, urine volume and water balance gap (fluid intake – urine volume) in Sprague-Dawley (SD) rats on a 0.2% NaCl diet with tap water to drink (NS+tap) or a 4% NaCl diet with saline to drink (HS+saline) (n=6 per group). (B) Fluid intake, urine volume and water balance gap in SD rats on NS+tap or a 4% NaCl diet with tap water to drink (HS+tap) (n=6 or 7 per group). (C) Fluid intake, urine volume and water balance gap in sham-operated Dahl salt-sensitive (DSS) rats on a 0.2% NaCl diet with tap water to drink (sham+NS+tap) or a 4% NaCl diet with saline to drink (sham+HS+saline) and renal-denervated DSS rats on a 4% NaCl diet with saline to drink (RDX+HS+saline) (n=6 per group). All values are expressed as the mean ± SD. *P < 0.05, **P < 0.01, versus NS+tap.



Supplementary figure 2. The effect of high salt intake on renal sympathetic nerve activity and energy expenditure.

(A) High salt intake suppresses renal sympathetic nerve activity (RSNA), which reduces heart rate (HR) and cardiovascular energy expenditure to compensate for the high salt-driven catabolic state in normal control rats. (B) High salt intake does not change HR and cardiovascular energy expenditure due to the abnormally activated RSNA in salt-sensitive rats.

	0.2% NaCl diet	4% NaCl diet
Energy content (kcal/g chow)	3.6	3.5
Protein (% of wet weight)	23.2	22.3
Protein (% of kcal intake)	25.7	25.7
Fat (% of wet weight)	5.1	4.9
Fat (% of kcal intake)	12.8	12.8
Nitrogen-free extracts (% of wet weight)	55.5	53.3
Nitrogen-free extracts (% of kcal intake)	61.5	61.5
Ingredients (% of wet weight chow)		
Sodium chloride	0.2	4.0
Potassium	0.9	0.9
Calcium	1.1	1.0
Magnesium	0.2	0.2
Phosphate	0.8	0.8

Supplementary table 1. Caloric content and ingredients in the 0.2% and 4% NaCl chow.