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# Reduced salt intake partially restores the circadian rhythm of bladder clock genes in Dahl salt-sensitive rats

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ABSTRACT

*Aims:* To examine the circadian expression changes in bladder clock genes in Dahl salt-sensitive rats following high salt intake.
*Main methods:* Eighteen rats were divided into three groups: the high-salt diet group (HS group), the normal-salt diet group (NS group), and the salt-load interruption group (from a 4 % salt diet to a normal diet; salt-load interruption group [SI group]). Each rat was placed in an individual metabolic cage for 24 h twice weekly. Water intake, urine production, voiding frequency, and voided volume per micturition were recorded. Furthermore, 108 control rats were prepared. Bladders were harvested every 4 h at six time points. Furthermore, the mRNA expression of clock genes and mechanosensors was analyzed.
*Key findings:* In the HS group, the bladder clock genes showed lower mRNA levels than in the NS group. The amplitude of circadian expression changes in bladder clock genes in the HS group was lower than that in the NS group. However, after changing from a 4 % salt diet to a normal diet, the waveforms of the clock gene expression in the SI group were closer to those of the NS group. The 24-h water intake and urinary volume of the SI group decreased to levels comparable to those of the NS group.
*Significance:* Reduced salt intake partially restored the circadian rhythms of bladder clock genes.

## 1. Introduction

Nocturia is caused by several factors, such as bladder storage dysfunction, nocturnal polyuria, and sleep disorders. Nocturnal polyuria is the most frequent cause of nocturia and is associated with hypertension secondary to excessive salt intake. Clinical studies have reported that nocturnal polyuria is primarily associated with high salt intake and is treated with salt restriction [1,2]. However, few studies have been conducted to determine whether salt restriction improves nocturnal polyuria.

Recently, some studies have reported that lower urinary tract functions are regulated by clock genes [3,4]. The circadian clock system plays an important role in the daily physiological processes. The suprachiasmatic nucleus of the brain functions as a master pacemaker. Moreover, it synchronizes peripheral clock gene rhythms present in tissues throughout the body, including the bladder [5,6]. The clock gene products create oscillations in sleep-awake rhythms and the gene expression of various metabolic enzymes, channels, and receptors with circadian rhythms. Okamura et al. [7] reported the molecular mechanism of the circadian rhythm as a core loop. The mechanism takes approximately 24 h and forms rhythms in various biological processes in the body.

Circadian rhythms are closely related to temperature and light [8]. As the temperature sensor and the factor responsible for bladder function, transient receptor potential vanilloid (TRPV) channels exist as multifunctional sensors. TRPV1 has been reported to be required for the detection of bladder stretch, which involves stretch-induced release of adenosine triphosphate (ATP) and nitric oxide [9]. TRPV4 senses distension of the bladder urothelium and contributes to bladder function [10]. Piezo1 is present in the bladder urothelium and senses bladder extension [11]. The vesicular nucleotide transporter (VNUT) is also expressed in the bladder urothelium. Urothelial VNUT-dependent ATP exocytosis is associated with bladder relaxation during the early stages of filling [12].

Approximately 80 % of nocturia cases are nocturnal polyuria [13,14]. This suggests that nocturnal awakening due to nocturnal

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polyuria may disturb clock genes in the bladder, leading to further lower urinary tract dysfunction [15]. Dahl salt-sensitive rats have been widely used to study salt-induced hypertension [16]. We recently reported that Dahl salt-sensitive rats fed a 2 % salt diet can be used as a model of nocturnal polyuria [17].

The circadian rhythm of clock gene expression in Dahl salt-sensitive rats has been reported in some organs. For example, Mohri et al. [18] reported that the amplitudes of circadian expression changes of clock genes in the heart, liver, and kidneys were significantly decreased in Dahl salt-sensitive rats fed a 4 % salt diet compared to a normal-salt diet. However, few reports have described the effect of lower urinary tract symptoms, such as nocturia and nocturnal polyuria, on clock gene expression in Dahl salt-sensitive rats.

In this study, our objective was to examine the circadian expression changes in bladder clock genes and their reversibility in Dahl salt-sensitive rats after high salt intake.

## 2. Materials and methods

### 2.1. Metabolic cage experiments

Eighteen six-week-old male Dahl salt-sensitive rats (SLC Inc., Shizuoka, Japan) were used. All rats were maintained under a 12-hour light/dark cycle (lights turned on automatically at 08:00 local time) with water and laboratory food ad libitum. The rats were divided into three groups; the high-salt diet group (HS group), the normal-salt diet group (NS group), and the salt-load interruption group (SI group) ($n = 6$ each, $n = 18$ in total). In the HS group, rats were fed a 4 % salt diet (4 % NaCl; CE-2, CLEA Japan, Inc., Tokyo, Japan) for 5 weeks. In the NS group, rats were fed a normal 0.3 % salt diet (0.3 % NaCl; CE-2, CLEA Japan, Inc., Tokyo, Japan) for 5 weeks. In the SI group, rats were fed a 4 % salt diet (4 % NaCl; CE-2, CLEA Japan, Inc., Tokyo, Japan) for 5 weeks and were fed a normal 0.3 % salt diet (0.3 % NaCl; CE-2, CLEA Japan, Inc., Tokyo, Japan) from weeks 6 to 11. In three groups, each rat was placed in an individual metabolic cage for 24 h for measurement twice a week at 1, 3, and 5 weeks after beginning the experiment. Furthermore, in the SI group, each rat was placed in an individual metabolic cage for 24 h for measurement twice a week at weeks 7, 9, and 11.

Food and water intake was recorded. Urine output was monitored every 2 min for 24 h with a KN-665FZiWP digital balance (Natsume Inc., Tokyo, Japan). The balance was located below the metabolic cage and connected to a computer using WinCT-Plus (A & D, Tokyo, Japan) for data collection. The parameters analyzed were 24-hour urinary volume, 24-hour voiding frequency, and voided volume per micturition. All urine samples were collected from vessels placed under the metabolic cages and weighed over time using electronic scales. The voided volume per micturition was calculated based on the change in the total amount of urine associated with the micturition. Because rats are nocturnal, the dark phase was defined as the active phase and the light phase as the sleep phase.

Body weight and blood pressure were measured before each metabolic cage experiment. Using the tail-cuff method, the hemodynamic parameters of the rats were analyzed using a BP-98A-L Blood Pressure Analysis System (Softron, Inc., Tokyo, Japan). Systolic blood pressure, diastolic blood pressure, and heart rate were recorded.

### 2.2. RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

In addition, we prepared more six-week-old male Dahl salt-sensitive rats ($n = 108$, SLC Inc., Shizuoka, Japan). Similar to the metabolic cage experiments, rats were divided into three groups following the HS group, NS group, and SI group ($n = 36$ each). Those in the HS group were fed a 4 % salt diet for 5 weeks. Those in the NS group were fed a normal 0.3 % salt diet for 5 weeks. Those in the SI group were fed a 4 % salt diet for 5 weeks, then a normal 0.3 % salt diet from weeks 6 to 11. All rats were maintained under a 12-h light/dark cycle (lights on from 08:00 to 20:00) with free access to water and laboratory food. The time at which the light phase began was set to the zeitgeber time (ZT) 0, with the dark phase beginning at ZT 12.

After the protocol, the rats were anesthetized with isoflurane (Escain ®; Mylan, Tokyo, Japan). Bladders were collected every 4 h at six time points (ZT 0, 4, 8, 12, 16, and 20) from the three groups ($n = 6$ for each time point, total $n = 36$ for each group). For qRT-PCR, after homogenization of the bladders, total RNA was extracted using an RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. We performed cDNA conversion using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time RT-PCR was performed using a StepOne Plus™ Real-time PCR system (Applied Biosystems) with TB Green® Premix Ex Taq (Takara Bio, Inc., Japan). The mRNA levels of *Bmal1*, *Clock*, *Per2*, *Cry2*, *TRPV1*, *TRPV4*, *Piezo1*, and *VNUT* were measured. Gene-specific primers and *β-actin* as a housekeeping gene are shown in Table 1. The results were analyzed using StepOne software (version 2.0; Applied Biosystems). All experiments were performed in accordance with institutional guidelines approved by the Nara Medical University Institutional Animal Care and Use Committee.

### 2.3. Statistical analysis

Statistical analyses were performed using the PRISM software version 9.3.1 (GraphPad Software, Inc., San Diego, CA, USA). All data were expressed as the median [25 % percentile, 75 % percentile].

Statistical significance was established at $P < 0.05$. For the metabolic cages data analysis of specific weeks and the PCR data analysis, one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used. To evaluate the independent predictive variable for clock genes and mechanosensory genes in the bladder, multiple linear regression analysis was used. Explanatory variables for clock genes included urinary volume in active or sleep phase, median voided volume per micturition in active or sleep phase, time (ZT 0, 4, 8, 12, 16, and 20), and food (4 % salt diet, normal-salt diet, and salt-load interruption). Explanatory variables for mechanosensors included clock genes (*Bmal1*, *Clock*, *Cry2*, and *Per2*), median voided volume per micturition in active or sleep phase, time (ZT 0, 4, 8, 12, 16, and 20), and food (4 % salt diet, normal-salt diet, and salt-load interruption). Urinary volume and median voided volume per micturition data was used the data of rats in metabolic cage experiments. The rats of RT-PCR and those in the metabolic cage experiments were different individuals, but we analyzed week age, time, and food in correspondence.

**Table 1**
The information of gene-specific primers and *β-actin* as a housekeeping gene.

| Gene | Accession No. | | Primers |
|---|---|---|---|
| *Bmal1* | NM024362 | F | CCGTGGACCAAGGAAGTAGA |
| | | R | CTGTGAGCTGTGGGAAGGTT |
| *Clock* | NM021856 | F | GCCGCAGAATAGCACCCAGAGT |
| | | R | ACTTGGCACCATGACGGCCC |
| *Per2* | NM031678 | F | GACGGGTCGAGCAAAGGA |
| | | R | GGGAAAAGTCCACATATCCATTCA |
| *Cry2* | NM133405.2 | F | GGGAGCATCAGCAACACAG |
| | | R | GCTTCCAGCTTGCGTTTG |
| *TRPV1* | NM031982.1 | F | GCTCTGCTCCTGGACGTT |
| | | R | GGCAATGTGCAGTGCTGT |
| *TRPV4* | NM023970 | F | ACTGGCAAGATCGGGGTCTT |
| | | R | GAGGAGAGGTCGTAGAGAGAAGAAT |
| *Piezo1* | NM001077200 | F | GACGCCTCACAAGGAAAGC |
| | | R | GGGCAGCATCTATGTCATCC |
| *VNUT* | NM001108613.1 | F | CTTGCTCTGGGTGTACTACGTG |
| | | R | AGGGCCAGGACAAGGTCT |
| *βactin* | NM031144 | F | TCTTCCAGCCTTCCTTCCTG |
| | | R | CCTGCTTGCTGATCCACATC |

## 3. Results

### 3.1. Physiological parameters in metabolic cages

Systolic blood pressure gradually increased in the HS and SI groups compared to that in the NS group. At 5 weeks after initializing the salt diets, systolic blood pressure was significantly higher in the HS and SI groups than in the NS group (HS: 167.3 [154.8, 222.5], NS: 132.0 [128.3, 135.8], SI: 192.8 [182.5, 194.1] mmHg, $P < 0.05$; Fig. 1A). After changing the diet from a 4 % salt diet to a normal 0.3 % salt diet, the systolic blood pressure remained high in the SI group. Subsequently, it gradually decreased but remained relatively high even at 11 weeks after initializing the salt diets. The pressure did not differ significantly between 5 and 11 weeks in the SI group (192.8 [182.5, 194.1] mmHg and 167.8 [151.8, 186.9] mmHg).

The 24-hour water intake increased in the HS and SI groups compared to that in the NS group on the first day of initiating the different diets for each group (Fig. 1B). This variance remained consistent 5 weeks after initializing the diets (HS: 58.6 [50.9, 62.7], NS: 30.4 [23.6, 38.7], SI: 48.2 [41.5, 63.2] mL, $P < 0.05$). However, after changing the diet from a 4 % salt diet to a normal 0.3 % salt diet, the 24-hour water intake in the SI group decreased to a level comparable to that of the NS group. There was no significant difference between the SI group at week 11 and the NS group at week 5 (NS: 30.4 [23.6, 38.7], SI: 21.3 [19.3, 26.8] mL). A similar trend was in the sum of urinary volume in both active and sleep phases. The urinary volume in the active phase was higher in the HS and SI groups than in the NS group from the first day of providing the different diets (HS: 28.3 [23.0, 33.5], NS: 8.25 [7.64, 12.2], SI: 23.8 [18.3, 35.9] mL, $P < 0.05$, 5 weeks; Fig. 1C). After changing the diet, urinary volume in the SI group in the active phase decreased to approximately the same level as that in the NS group. There was no significant difference between the SI group at week 11 and the NS group at week 5 (NS: 8.25 [7.64, 12.2] mL, SI: 7.00 [6.03, 8.83] mL). The urinary volume in the sleep phase was also higher in the HS and SI groups than in the NS group from the first day of providing the different diets (HS: 12.4 [9.32, 15.4], NS: 5.83 [4.63, 7.10], SI: 10.3 [8.42, 12.6] mL, $P < 0.05$, 5 weeks; Fig. 1D). After changing the diet, the urinary volume in the SI group in the sleep phase also decreased to a level comparable to that in the NS group. The urinary volume in the sleep phase in the SI group at week 11 was lower than that in the NS group at week 5 (NS: 5.83 [4.63, 7.10] mL SI: 3.84 [3.32, 5.02] mL, $P < 0.05$).

A similar trend was also found in voided volume per micturition in both the active and sleep phases. The voided volume per micturition in the active phase was higher in the HS and SI groups than in the NS group from the first day of providing the different diets (HS: 0.71 [0.68, 0.76] mL, NS: 0.40 [0.34, 0.42] mL, SI: 0.81 [0.75, 0.98] mL, $P < 0.05$, 5 weeks; Fig. 1E). After changing the diet, the voided volume per micturition in the SI group in the active phase decreased to a level comparable to that in the NS group. There was no significant difference in these values between the SI group at week 11 and the NS group at week 5 (NS: 0.40 [0.34, 0.42], mL, CS: 0.39 [0.35, 0.42] mL). The voided volume per micturition in the sleep phase was also higher in the HS and SI groups than in the NS group from the first day of providing the different diets (HS: 1.25 [1.10, 1.35] mL, NS: 0.70 [0.57, 0.89] mL, SI: 1.36 [1.28, 1.41] mL, $P < 0.05$, 5 weeks; Fig. 1F). After changing the diet, the voided volume per micturition in the SI group in the sleep phase decreased to approximately the same level as in the NS group. There was no significant difference between the SI group at week 11 and the NS group at week 5 (NS: 0.70 [0.57, 0.89] mL, SI: 0.57 [0.47, 0.92] mL). Urinary frequency in the active phase at week 5 was higher in the HS group than in the NS group (HS: 39.0 [35.3, 43.8] times, NS: 23.5 [20.3, 30.0] times, SI: 32.0 [20.0, 44.0] times, $P < 0.05$; Fig. 1G). After changing the diet, urinary frequency in the SI group in the active phase decreased to a level comparable to that in the NS group. There was no

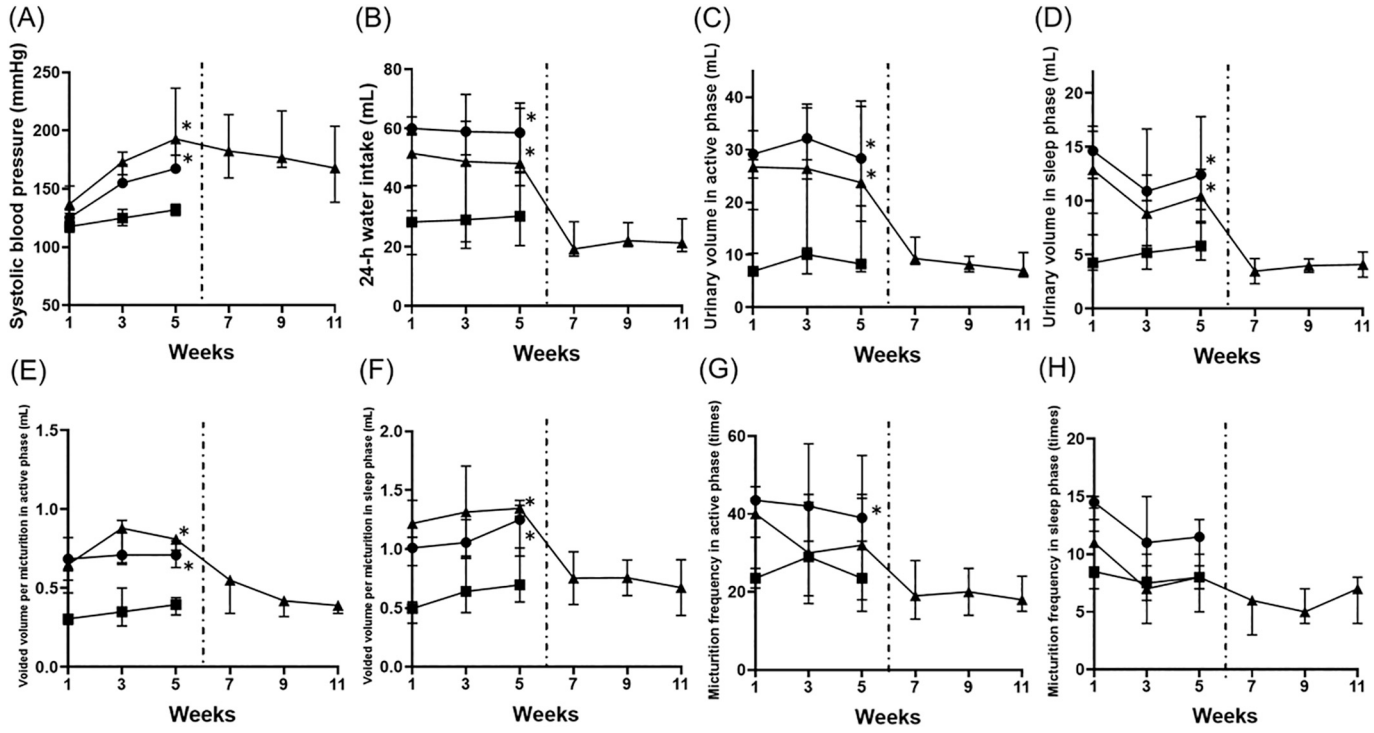


**Fig. 1.** Parameters showed changes from weeks 1 to 5 or week 11 in metabolic cages: (A) mean systolic blood pressure; (B) 24-hour water intake; (C) urinary volume in the active phase; (D) urinary volume in the sleep phase; (E) voided volume per micturition in the active phase; (F) voided volume per micturition in the sleep phase; (G) micturition frequency in the active phase; (H) micturition frequency in the sleep phase. The vertical dashed line in these panels is the timing of diet change in the SI group. The median and error values for each group are presented. ●filled circle: the high-salt diet group (HS group); ■filled square: the normal-salt diet group (NS group); ▲filled triangle: the salt-load interruption group (SI group) * $P < 0.05$, compared to NS group at week 5 Dunnett's multiple comparison test was used.

significant difference between the SI group at week 11 and the NS group at week 5 (NS: 23.5 [20.3, 30.0] times, SI: 18.0 [16.5, 22.5] times). On the other hand, urinary frequency in the sleep phase at week 5 did not differ among three groups (HS: 11.5 [7.8, 12.3] times, NS: 8.0 [7.8, 9.0] times, SI: 8.0 [6.0, 9.5] times; Fig. 1H). After changing the diet, urinary frequency in the SI group in the active phase was almost same as that in the NS group. There was no significant difference between the SI group at week 11 and the NS group at week 5 (NS: 8.0 [7.8, 9.0] times, SI: 7.0 [5.0, 7.5] times).

In order to analyze the ratio of urinary volume in the sleep phase, we calculated the diurnal polyuria index (DPi) in rats (DPi refers to the ratio of diurnal urinary volume to daily urinary volume), which was used as a corresponding index for nocturnal polyuria in humans. However, DPi at week 5 did not differ significantly among the three groups (HS: 29.5 [27.1, 33.6]%, NS: 38.5 [32.5, 44.9]%, SI: 29.3 [23.3, 38.6]%). After changing the diet, there was no significant difference between the SI group at week 11 and the NS group at week 5 (SI: 35.4 [33.1, 38.6]%, NS: 38.5 [32.5, 44.9]%).

### 3.2. Clock gene expression

In the bladder, the four clock genes (*Bmal1*, *Clock*, *Per2*, and *Cry2*) in the HS group showed lower levels of mRNA than those in the NS and SI groups (Fig. 2A–D). In the three groups, the expression of *Bmal1* and *Clock* exhibited circadian rhythms with peaks in the sleep phase (ZT 4) and nadirs in the active phase (ZT 12–16; Fig. 2A, B). In contrast, *Per2* and *Cry2* expression had reverse circadian rhythms with peaks in the active phase (ZT 12–16) and nadirs in the sleep phase (ZT 4; Fig. 2C, D). The amplitudes of circadian expression changes in the clock genes (*Bmal1 and Per2*) in the HS group was lower than those in the NS and SI groups (Fig. 2A, D).

To investigate the relationship between these clock genes and urinary parameters in the metabolic cage, multiple linear regression analysis was performed using the mRNA levels of *Bmal1*, *Clock*, *Per2*, and *Cry2* as the dependent variables (Table 2). In this analysis, the clock genes were affected by time and food (presence or absence of salt-load), but they were not affected by urinary volume and voided volume per micturition.

### 3.3. Mechanosensors and VNUT gene expression

In the bladder, all mechanosensors (*TRPV1*, *TRPV4*, and *Piezo1*) and *VNUT* genes in the HS group showed lower levels of mRNA expression than those in the NS and SI groups (Fig. 3A-D). In the NS and SI groups, the expression of these genes had a circadian rhythm, whereas the expression in the HS group did not have a circadian rhythm and showed an almost flattened pattern.

To investigate the relationship between these genes and urinary parameters in the metabolic cage, multiple linear regression analysis was performed using the mRNA levels of *TRPV1*, *TRPV4*, *Piezo1*, and *VNUT* as the dependent variable (Table 3). In this analysis, the mechanosensors and *VNUT* genes were affected by clock genes, but were not affected by voided volume per micturition, time, and food (presence or absence of salt-load).

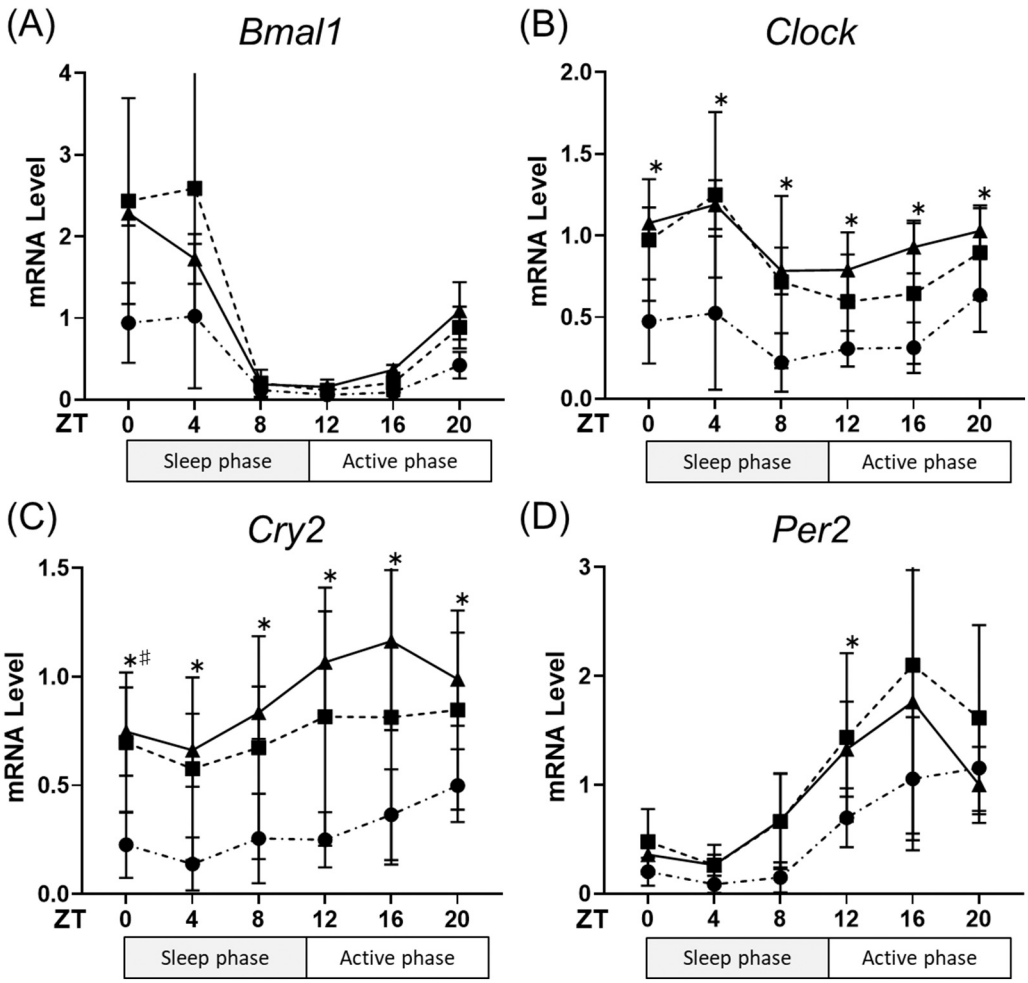


**Fig. 2.** Changes in clock gene mRNA expression in bladders: (A) *Bmal1* (brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1); (B) *Clock* (circadian locomotor output cycle kaput); (C) *Cry2* (cryptochrome 2); (D) *Per2* (period 2); ZT: zeitgeber time. The median and error values for each group are presented. ●filled circle: the high-salt diet group (HS group); ■filled square: the normal-salt diet group (NS group); ▲filled triangle: the salt-load interruption group (SI group). # $P < 0.05$, HS group compared to NS group * $P < 0.05$, HS group compared to SI group Dunnett's multiple comparison test was used.

**Table 2**
The multiple linear regression analysis using the mRNA levels of *Bmal1*, *Clock*, *Per2*, and *Cry2* as the dependent variables.

| | Bmal1 | | | |
|---|---|---|---|---|
| | Regression coefficient | 95 % confidence interval | | *P* value |
| Urine volume | 0.12 | 0.02 | 0.22 | 0.016 |
| Voided volume per micturition | 3.24 | −0.75 | 7.22 | 0.11 |
| Time | | | | |
| ZT0 | 1.00 | | | |
| ZT4 | −0.11 | −0.49 | 0.28 | 0.58 |
| ZT8 | −1.72 | −2.10 | −1.33 | <0.0001 |
| ZT12 | −1.53 | −2.38 | −0.68 | 0.0006 |
| ZT16 | −1.42 | −2.27 | −0.57 | 0.0013 |
| ZT20 | −0.84 | −1.70 | 0.0092 | 0.053 |
| Food | | | | |
| High-salt diet | 1.00 | | | |
| Normal-salt diet | 3.63 | 0.65 | 6.61 | 0.017 |
| Salt-load interruption | 3.93 | 0.55 | 7.32 | 0.023 |

| | Clock | | | |
|---|---|---|---|---|
| | Regression coefficient | 95 % confidence interval | | P value |
| Urine volume | 0.041 | −0.0066 | 0.088 | 0.091 |
| Voided volume per micturition | 1.14 | −0.83 | 3.11 | 0.25 |
| Time | | | | |
| ZT0 | 1.00 | | | |
| ZT4 | 0.15 | −0.043 | 0.34 | 0.13 |
| ZT8 | −0.27 | −0.46 | −0.077 | 0.0063 |
| ZT12 | −0.18 | −0.60 | 0.24 | 0.39 |
| ZT16 | −0.12 | −0.54 | 0.30 | 0.58 |
| ZT20 | 0.11 | −0.31 | 0.5300 | 0.62 |
| Food | | | | |
| High-salt diet | 1.00 | | | |
| Normal-salt diet | 1.48 | 0.0057 | 2.95 | 0.049 |
| Salt-load interruption | 1.74 | 0.066 | 3.41 | 0.042 |

| | Cry2 | | | |
|---|---|---|---|---|
| | Regression coefficient | 95 % confidence interval | | P value |
| Urine volume | 0.019 | −0.035 | 0.074 | 0.49 |
| Voided volume per micturition | 1.12 | −1.13 | 3.38 | 0.33 |
| Time | | | | |
| ZT0 | 1.00 | | | |
| ZT4 | −0.098 | −0.32 | 0.12 | 0.37 |
| ZT8 | 0.031 | −0.19 | −1.33 | 0.78 |
| ZT12 | 0.40 | −0.084 | 0.88 | 0.1 |
| ZT16 | 0.47 | −0.014 | 0.95 | 0.057 |
| ZT20 | 0.47 | −0.016 | 0.95 | 0.058 |
| Food | | | | |
| High-salt diet | 1.00 | | | |
| Normal-salt diet | 1.19 | −0.50 | 2.88 | 0.16 |
| Salt-load interruption | 1.47 | −0.45 | 3.38 | 0.13 |

| | Per2 | | | |
|---|---|---|---|---|
| | Regression coefficient | 95 % confidence interval | | P value |
| Urine volume | −0.071 | −0.17 | 0.032 | 0.17 |
| Voided volume per micturition | −2.21 | −6.44 | 2.03 | 0.30 |
| Time | | | | |
| ZT0 | 1.00 | | | |
| ZT4 | −0.14 | −0.55 | 0.27 | 0.49 |
| ZT8 | 0.15 | −0.26 | 0.56 | 0.47 |
| ZT12 | 0.56 | −0.34 | 1.47 | 0.22 |
| ZT16 | 1.05 | 0.14 | 1.95 | 0.024 |
| ZT20 | 0.67 | −0.24 | 1.57 | 0.15 |
| Food | | | | |
| High-salt diet | 1.00 | | | |
| Normal-salt diet | −1.37 | −4.53 | 1.79 | 0.39 |
| Salt-load interruption | −1.82 | −5.42 | 1.77 | 0.32 |

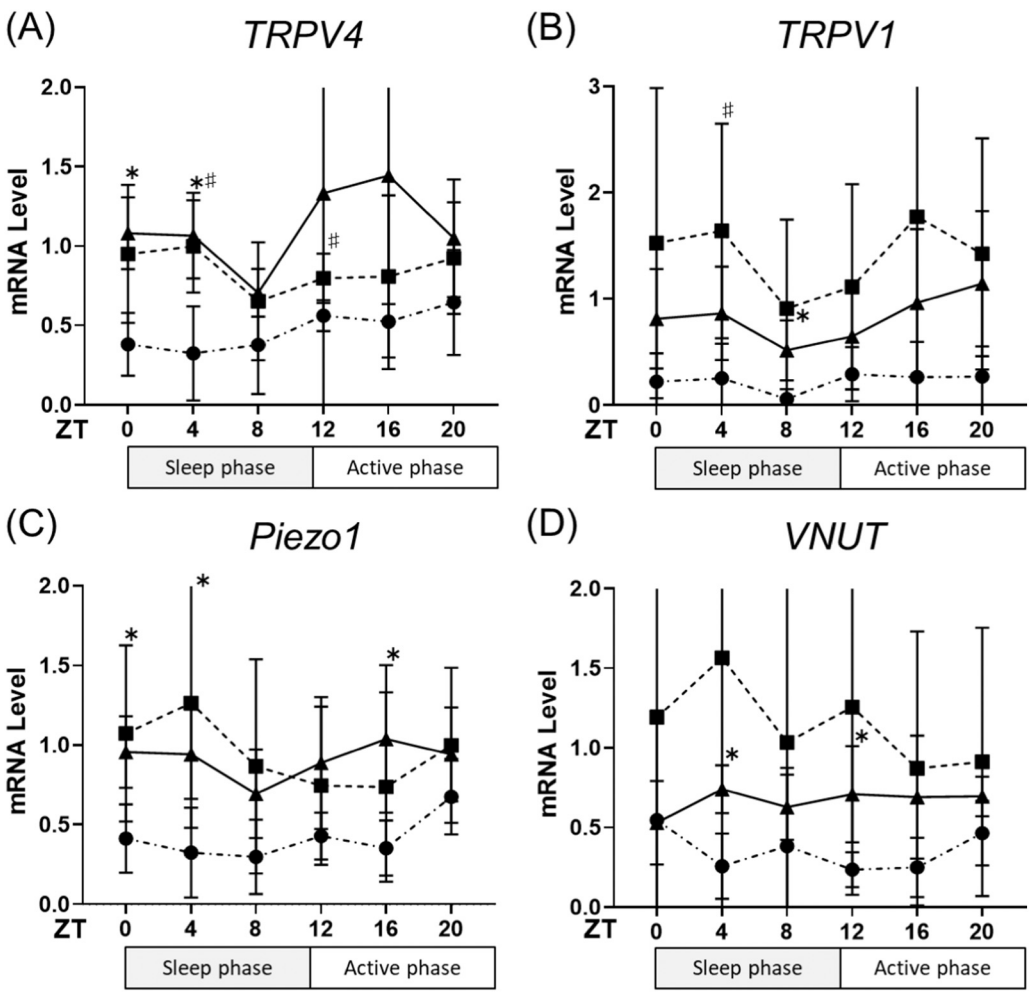


**Fig. 3.** Changes in mechanosensors and *VNUT* genes mRNA expression in bladders: (A) *TRPV4* (transient receptor potential vanilloid channel 4); (B) *TRPV1* (transient receptor potential vanilloid channel 1); (C) *Piezo1*; (D) *VNUT* (vesicular nucleotide transporter); ZT: zeitgeber time. The median and error values for each group are presented. ●filled circle: the high-salt diet group (HS group); ■filled square: the normal-salt diet group (NS group); ▲filled triangle: the salt-load interruption group (SI group). # $P < 0.05$, HS group compared to NS group * $P < 0.05$, HS group compared to SI group Dunnett's multiple comparison test was used.

## 4. Discussion

We revealed a relationship between polyuria due to high salt intake and clock gene expression in Dahl salt-sensitive rats. To the best of our knowledge, this study is the first to reveal that polyuria and nocturnal polyuria due to high salt intake disturb the waveforms of clock gene expression in the bladder and that a reduced salt diet rapidly restores polyuria and nocturnal polyuria, as well as the waveforms of clock gene expression.

Among the various types of clock genes, *Clock*, *Bmal1*, *Cry*, and *Per* have been supposed to play the most important roles in regulating circadian rhythms. Circadian *Bmal1* expression appears to be positively controlled by Per and Cry proteins, and *Bmal1* transcription may be negatively regulated by *Bmal1* and *Clock* [19]. In this study, *Bmal1* and *Clock* expression in the bladder had circadian rhythms with peaks in the sleep phase (ZT4) and nadirs in the active phase (ZT 12–16) in all groups. In contrast, *Per2* and *Cry2* expression in the bladder had reverse circadian rhythms with peaks in the active phase (ZT 12–16) and nadirs in the sleep phase (ZT 4) in all groups. These results can be explained by mechanisms such as positive and negative feedbacks. Furthermore, the amplitudes of circadian expression changes of the clock genes (*Bmal1* and *Per2*) in Dahl salt-sensitive rats fed a 4 % salt diet was lower than in rats fed a normal-salt diet. This result was comparable to that of Mohri et al. [18], who showed that the amplitudes of circadian expression changes of clock genes (*mPer2*, *Bmal1*, and *dbp*) in the heart, liver, and kidney were significantly decreased in Dahl salt-sensitive rats fed a high-salt diet compared with rats fed a normal-salt diet. One possible explanation is that high urinary volume due to high salt intake might affect sympathetic and/or parasympathetic nervous activity in the bladder because the autonomic nervous system participates in pathways that affect the central and peripheral clock systems [20]. Another reason is that the amplitude of clock genes in the bladder depends on changes in the expression of central clock genes. Mohri et al. [18] reported that a high-salt diet and/or high blood pressure might alter the expression of clock genes in the suprachiasmatic nucleus (SCN). The SCN plays the role of the mammalian central clock and affects the input signaling pathways for peripheral oscillators. Further studies are necessary to clarify the expression levels of clock genes in the SCN. Ihara et al. [21] reported that *clock*-mutant mice showed a phenotype of nocturia and nocturnal polyuria. In the study, abnormalities in clock genes might induce nocturia and nocturnal polyuria as one of several causes. This possibility also applies to our study. To investigate the correlation between clock genes and urinary parameters in the metabolic cage, we performed multiple linear regression analysis. We considered the possibility that clock genes were disturbed by nocturia and nocturnal polyuria regardless of time or food, but clock genes were significantly affected by time and food (salt load), but they were not affected by urinary volume and voided volume per micturition that reflects bladder hyperextension due to polyuria. Therefore, the regulatory systems of clock genes lead to new insights into the pathophysiology of nocturia and nocturnal polyuria.

Notably, by reducing salt intake, the waveforms of the clock gene expression in the SI group were closer to those in the NS group. In this study, after changing the diet from a 4 % salt diet to a normal 0.3 % salt diet, the 24-hour water intake immediately decreased to a level comparable to that of the NS group. A similar trend was found in the sum of

**Table 3**
The multiple linear regression analysis using the mRNA levels of *TRPV1*, *TRPV4*, *Piezo1*, and *VNUT* as the dependent variable.

| | TRPV4 | | | |
|---|---|---|---|---|
| | Regression coefficient | 95 % confidence interval | | P value |
| *Bmal1* | 0.0099 | −0.17 | 0.19 | 0.91 |
| *Clock* | 0.61 | 0.11 | 1.11 | 0.017 |
| *Cry2* | 0.18 | −0.28 | 0.64 | 0.45 |
| *Per2* | 0.17 | −0.022 | 0.36 | 0.082 |
| Voided volume per micturition | 0.24 | −0.78 | 1.25 | 0.64 |
| Time | | | | |
| ZT0 | 1.00 | | | |
| ZT4 | −0.055 | −0.33 | 0.22 | 0.69 |
| ZT8 | −0.077 | −0.43 | 0.28 | 0.67 |
| ZT12 | 0.20 | −0.35 | 0.74 | 0.48 |
| ZT16 | 0.088 | −0.45 | 0.63 | 0.75 |
| ZT20 | −0.042 | −0.53 | 0.45 | 0.87 |
| Food | | | | |
| High-salt diet | 1.00 | | | |
| Normal-salt diet | 0.048 | −0.44 | 0.53 | 0.84 |
| Salt-load interruption | 0.25 | −0.32 | 0.83 | 0.39 |

| | TRPV1 | | | |
|---|---|---|---|---|
| | Regression coefficient | 95 % confidence interval | | P value |
| *Bmal1* | 0.57 | 0.35 | 0.79 | <0.0001 |
| *Clock* | −0.73 | −1.33 | −0.13 | 0.018 |
| *Cry2* | 0.86 | 0.30 | 1.42 | 0.0030 |
| *Per2* | 0.67 | 0.43 | 0.90 | <0.0001 |
| Voided volume per micturition | −0.019 | −1.25 | 1.21 | 0.98 |
| Time | | | | |
| ZT0 | 1.00 | | | |
| ZT4 | 0.41 | 0.08 | 0.74 | 0.015 |
| ZT8 | 0.30 | −0.13 | 0.73 | 0.17 |
| ZT12 | −0.035 | −0.69 | 0.62 | 0.92 |
| ZT16 | −0.12 | −0.77 | 0.54 | 0.72 |
| ZT20 | −0.081 | −0.67 | 0.51 | 0.79 |
| Food | | | | |
| High-salt diet | 1.00 | | | |
| Normal-salt diet | 0.38 | −0.21 | 0.960 | 0.20 |
| Salt-load interruption | −0.068 | −0.76 | 0.63 | 0.85 |

| | Piezo1 | | | |
|---|---|---|---|---|
| | Regression coefficient | 95 % confidence interval | | P value |
| *Bmal1* | 0.14 | 0.062 | 0.22 | 0.0005 |
| *Clock* | 0.38 | 0.17 | 0.59 | 0.0005 |
| *Cry2* | 0.72 | 0.52 | 0.92 | <0.0001 |
| *Per2* | 0.017 | −0.065 | 0.098 | 0.69 |
| Voided volume per micturition | −0.28 | −0.71 | 0.150 | 0.20 |
| Time | | | | |
| ZT0 | 1.00 | | | |
| ZT4 | 0.061 | −0.055 | 0.18 | 0.30 |
| ZT8 | 0.12 | −0.030 | 0.27 | 0.12 |
| ZT12 | 0.0077 | −0.22 | 0.24 | 0.95 |
| ZT16 | −0.069 | −0.30 | 0.16 | 0.56 |
| ZT20 | −0.064 | −0.27 | 0.14 | 0.54 |
| Food | | | | |
| High-salt diet | 1.00 | | | |
| Normal-salt diet | −0.17 | −0.380 | 0.033 | 0.099 |
| Salt-load interruption | −0.38 | −0.63 | −0.14 | 0.0026 |

| | VNUT | | | |
|---|---|---|---|---|
| | Regression coefficient | 95 % confidence interval | | P value |
| *Bmal1* | 0.29 | 0.073 | 0.51 | 0.0094 |
| *Clock* | 0.12 | −0.47 | 0.72 | 0.68 |
| *Cry2* | 1.71 | 1.16 | 2.26 | <0.0001 |
| *Per2* | −0.27 | −0.50 | −0.038 | 0.023 |
| Voided volume per micturition | 0.16 | −1.06 | 1.37 | 0.80 |
| Time | | | | |
| ZT0 | 1.00 | | | |
| ZT4 | 0.24 | −0.087 | 0.57 | 0.15 |

(*continued on next page*)

**Table 3** (*continued*)

| | VNUT | | | |
|---|---|---|---|---|
| | Regression coefficient | 95 % confidence interval | | P value |
| ZT8 | 0.44 | 0.017 | 0.87 | 0.042 |
| ZT12 | 0.54 | −0.11 | 1.18 | 0.10 |
| ZT16 | 0.38 | −0.27 | 1.02 | 0.25 |
| ZT20 | 0.17 | −0.42 | 0.75 | 0.57 |
| Food | | | | |
| High-salt diet | 1.00 | | | |
| Normal-salt diet | −0.0074 | −0.59 | 0.57 | 0.98 |
| Salt-load interruption | −0.8 | −1.49 | −0.11 | 0.023 |

urinary volume in both active and sleep phases. Therefore, we considered that this reduction in water intake and the sum of urinary volume helps to restore abnormal autonomic nervous activity in the bladder due to polyuria and that normalization of micturition represents a recovery of the expression of clock genes.

In addition, we examined the mechanosensors (*TRPV1*, *TRPV4*, and *Piezo1*) and *VNUT* genes in the bladder. All mechanosensors and *VNUT* genes in the high-salt diet group showed lower mRNA levels than those in the NS group. These results are comparable to those of previous studies. Ihara et al. [22] reported disturbed diurnal variation in *TRPV4*, *VNUT*, and *Piezo1* expression in the bladder mucosa of clock-mutant versus wild-type mice. The authors of the cited study proposed that *TRPV4*, *VNUT*, and *Piezo1* are regulated by clock genes and speculated that increased expression of *Cry2* and *Clock* during the active phase might be related to the expression levels of mechanosensors and *VNUT*. In our study, the expression levels of these genes in the NS and the SI groups had a waveform that resembled a circadian rhythm, but the expression in the HS group did not have a circadian rhythm and showed an almost flattened pattern. From this result, we speculated that frequent urination due to excessive salt intake leads to the disappearance of daily fluctuations and disturbance of the circadian rhythm. To examine the correlation between mechanosensors and *VNUT*, clock genes, and urinary parameters in the metabolic cage, we performed a multiple linear regression analysis. The mechanosensors and *VNUT* were significantly regulated by clock genes, but they were not affected by voided volume per micturition that reflects bladder hyperextension due to polyuria, time, and food (salt-load). This result is similar to that of a previous study [22], which supports the hypothesis that the mechanosensors and *VNUT* are regulated by clock genes.

In this study, the systolic blood pressure in Dahl salt-sensitive rats fed a 4 % salt diet was substantially higher than that in rats fed a normal-salt diet. After changing the diet from 4 % salt to normal 0.3 % salt, we speculated that systolic blood pressure would drop when the diet was changed. However, the pressure remained high. The reason for this was unclear, but we deduced that high salt intake caused irreversible changes, such as cardiovascular disease or chronic kidney disease. Some studies have reported that in Dahl salt-sensitive rats, systemic hypertension causes left ventricular hypertrophy and renal hypertrophy with glomerulosclerosis [23–25]. Therefore, these chronic irreversible changes may have resulted in high blood pressure persisting after salt restriction.

After changing the diet from a 4 % salt diet to a normal 0.3 % salt diet, the 24-hour water intake immediately decreased to a level comparable to the normal-salt group. According to this, a similar tendency was found in the sum of urinary volume in both active and sleep phases. These results indicate that salt reduction contributes to the reduction in water intake and the sum of the urinary volume. Matsuo et al. [2] reported that reducing salt intake may be effective in patients with lower urinary tract symptoms with nocturia due to excessive salt intake. Our study also shows a relationship between salt reduction and nocturia. In other basic studies, Yamamoto et al. [26] reported that high salt loading induced urinary storage dysfunction via the upregulation of epithelial sodium channel alpha in the bladder epithelium in Dahl salt-sensitive rats. In our study, we did not directly investigate upregulation of epithelial sodium channel alpha in the bladder epithelium, but speculated that polyuria and nocturia due to excessive salt intake are regulated by upregulation of epithelial sodium channel alpha.

This study has some limitations. First, no bladder urodynamic study was performed. Flores et al. [27] reported bladder overdistension in a high-salt diet group, as confirmed by cystometry, which was evident from a significantly higher bladder capacity and compliance. However, they did not observe differences in the detrusor contractility between the two groups. Therefore, we speculate that detrusor contractility in rats fed a 4 % salt diet is comparable to that of rats fed a normal diet. Second, we did not measure the normal-salt diet rat data at 11 weeks. However, we considered there was no significant difference between weeks 5 and 11 because the rats were fed a normal-salt diet. Third, the model was not an ideal model of nocturnal polyuria. The model fed with a 2 % salt diet were used in our previous study [17]. In the sleep phase, the rats that were fed a 2 % salt diet ingested approximately 10 % of the water that they ingested in 24 h and passed one-third of the normal urinary volume that they had passed in 24 h. It is important to note that the rats accumulated body water in the active phase and urinated greater volumes in the sleep phase. These findings are consistent with those of clinical patients with nocturnal polyuria in whom nighttime water intake is low. For this reason, we suggest that Dahl salt-sensitive rats that are fed a 2 % salt diet may be candidates for a nocturnal polyuria model. Forth, we did not check protein expression levels in the bladder. The path from mRNA translation into protein is complicated, and a previous study reported there is a 6-hour interval between mRNA expression of peripheral tissues and clock protein expression in the mouse liver [28]. However, the time difference between mRNA and protein expression in Dahl salt-sensitive rat pathology is unclear. Therefore, a clear priority in future studies will be to determine clock protein expression patterns in Dahl salt-sensitive rats.

## 5. Conclusion

In Dahl salt-sensitive rats fed a high-salt diet, bladder clock genes showed lower mRNA levels than those of rats fed a normal diet. The amplitude of circadian expression changes in bladder clock genes in rats fed a high-salt diet was lower than those in rats fed a normal diet. After salt-load interruption, the 24-hour water intake and urinary volume immediately decreased to levels comparable to the normal diet group, and reduced salt intake partially restored the circadian rhythms of the bladder clock genes. In multiple linear regression analysis, clock genes were affected by time and salt-load, but they were not affected by urinary volume and voided volume per micturition that reflects bladder hyperextension due to polyuria.

## CRediT authorship contribution statement

**Takashi Iwamoto:** Conceptualization, Methodology, Investigation, Writing – original draft, Visualization. **Kazumasa Torimoto:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Project administration. **Daisuke Gotoh:** Methodology, Investigation.

**Sayuri Onishi:** Investigation. **Shunta Hori:** Methodology, Investigation. **Yousuke Morizawa:** Methodology, Investigation. **Yasushi Nakai:** Methodology, Data curation. **Makito Miyake:** Methodology, Formal analysis. **Kiyohide Fujimoto:** Conceptualization, Writing – review & editing, Funding acquisition.

## Data availability

Data will be made available on request.

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## References

[1] T. Matsuo, Y. Miyata, H. Sakai, Daily salt intake is an independent risk factor for pollakiuria and nocturia, Int. J. Urol. 24 (2017) 384–389, https://doi.org/10.1111/iju.13321.
[2] T. Matsuo, Y. Miyata, H. Sakai, Effect of salt intake reduction on nocturia in patients with excessive salt intake, Neurourol. Urodyn. 38 (2019) 927–933, https://doi.org/10.1002/nau.23929.
[3] H. Negoro, A. Kanematsu, M. Doi, S.O. Suadicani, M. Matsuo, M. Imamura, et al., Involvement of urinary bladder connexin43 and the circadian clock in coordination of diurnal micturition rhythm, Nat. Commun. 3 (2012) 809, https://doi.org/10.1038/ncomms1812.
[4] T. Ihara, T. Mitsui, Y. Nakamura, S. Kira, H. Nakagomi, N. Sawada, et al., Clock genes regulate the circadian expression of Piezo1, TRPV4, connexin26, and VNUT in an ex vivo mouse bladder mucosa, PLOS ONE 12 (2017), e0168234, https://doi.org/10.1371/journal.pone.0168234.
[5] E.E. Zhang, S.A. Kay, Clocks not winding down: unravelling circadian networks, Nat. Rev. Mol. Cell Biol. 11 (2010) 764–776, https://doi.org/10.1038/nrm2995.
[6] C. Dibner, U. Schibler, U. Albrecht, The mammalian circadian timing system: organization and coordination of central and peripheral clocks, Annu. Rev. Physiol. 72 (2010) 517–549, https://doi.org/10.1146/annurev-physiol-021909-135821.
[7] H. Okamura, M. Doi, J.M. Fustin, Y. Yamaguchi, M. Matsuo, Mammalian circadian clock system: molecular mechanisms for pharmaceutical and medical sciences, Adv. Drug Deliv. Rev. 62 (2010) 876–884, https://doi.org/10.1016/j.addr.2010.06.004.
[8] M.O. Poletini, M.N. Moraes, B.C. Ramos, R. Jeronimo, Ana Maria de Lauro Castrucci, TRP channels: a missing bond in the entrainment mechanism of peripheral clocks throughout evolution, Temperature (Austin) 2 (2015) 522–534, https://doi.org/10.1080/23328940.2015.1115803.
[9] L.A. Birder, Y. Nakamura, S. Kiss, M.L. Nealen, S. Barrick, A.J. Kanai, et al., Altered urinary bladder function in mice lacking the vanilloid receptor TRPV1, Nat. Neurosci. 5 (2002) 856–860, https://doi.org/10.1038/nn902.
[10] T. Mochizuki, T. Sokabe, I. Araki, K. Fujishita, K. Shibasaki, K. Uchida, K. Naruse, S. Koizumi, M. Takeda, M. Tominaga, The TRPV4 cation channel mediates stretch-evoked Ca2+ influx and ATP release in primary urothelial cell cultures, J. Biol. Chem. 284 (2009) 21257–21264, https://doi.org/10.1074/jbc.M109.020206.
[11] T. Miyamoto, T. Mochizuki, H. Nakagomi, S. Kira, M. Watanabe, Y. Takayama, Y. Suzuki, S. Koizumi, M. Takeda, M. Tominaga, Functional role for Piezo1 in stretch-evoked Ca2+ influx and ATP release in urothelial cell cultures, J. Biol. Chem. 289 (2014) 16565–16575, https://doi.org/10.1074/jbc.M113.528638.
[12] H. Nakagomi, M. Yoshiyama, T. Mochizuki, T. Miyamoto, R. Komatsu, Y. Imura, et al., Urothelial ATP exocytosis: regulation of bladder compliance in the urine storage phase, Sci. Rep. 6 (2016) 29761, https://doi.org/10.1038/srep29761.
[13] J.L. Bosch, J.P. Weiss, The prevalence and causes of nocturia, J. Urol. 189 (Supplement) (2013) S86–S92, https://doi.org/10.1016/j.juro.2012.11.033.
[14] J.P. Weiss, P.E. van Kerrebroeck, B.M. Klein, J.P. Nørgaard, Excessive nocturnal urine production is a major contributing factor to the etiology of nocturia, J. Urol. 186 (2011) 1358–1363, https://doi.org/10.1016/j.juro.2011.05.083.
[15] T. Ihara, T. Mitsui, H. Shimura, S. Tsuchiya, M. Kanda, S. Kira, H. Nakagomi, N. Sawada, M. Kamiyama, E. Shigetomi, Y. Shinozaki, S. Koizumi, M. Takeda, Different effects of GsMTx4 on nocturia associated with the circadian clock and Piezo1 expression in mice, Life Sci. 278 (2021), 119555, https://doi.org/10.1016/j.lfs.2021.119555.
[16] J.P. Rapp, Dahl salt-susceptible and salt-resistant rats.A review, Hypertension 4 (1982) 753–763, https://doi.org/10.1161/01.hyp.4.6.753.
[17] T. Iwamoto, K. Torimoto, D. Gotoh, S. Hori, Y. Nakai, M. Miyake, Y. Tokita, R. Kobayashi, K. Aoki, K. Fujimoto, The effects of choreito on a model of nocturnal polyuria using Dahl salt-sensitive rats, Low. Urin. Tract Symptoms 14 (2022) 122–128, https://doi.org/10.1111/luts.12418.
[18] T. Mohri, N. Emoto, H. Nonaka, H. Fukuya, K. Yagita, H. Okamura, M. Yokoyama, Alterations of circadian expressions of clock genes in dahl salt-sensitive rats fed a high-salt diet, Hypertension 42 (2003) 189–194, https://doi.org/10.1161/01.HYP.0000082766.63952.49.
[19] W.J. Yu, M. Nomura, M. Ikeda, Interactivating feedback loops within the mammalian clock: BMAL1 is negatively autoregulated and upregulated by CRY1, CRY2, and PER2, Biochem. Biophys. Res. Commun. 290 (2002) 933–941, https://doi.org/10.1006/bbrc.2001.6300.
[20] T.J. Bartness, C.K. Song, G.E. Demas, SCN efferents to peripheral tissues: implications for biological rhythms, J. Biol. Rhythm. 16 (2001) 196–204, https://doi.org/10.1177/074873040101600302.
[21] T. Ihara, T. Mitsui, Y. Nakamura, S. Kira, T. Miyamoto, H. Nakagomi, et al., The clock mutant mouse is a novel experimental model for nocturia and nocturnal polyuria, Neurourol. Urodyn. 36 (2017) 1034–1038-2038, https://doi.org/10.1002/nau.23062.
[22] T. Ihara, T. Mitsui, Y. Nakamura, M. Kanda, S. Tsuchiya, S. Kira, et al., The circadian expression of Piezo1, TRPV4, connexin26, and VNUT, associated with the expression levels of the clock genes in mouse primary cultured urothelial cells, Neurourol. Urodyn. 37 (2018) 942–951, https://doi.org/10.1002/nau.23400.
[23] Y. Iwanaga, Y. Kihara, K. Hasegawa, K. Inagaki, T. Yoneda, S. Kaburagi, M. Araki, S. Sasayama, Cardiac endothelin-1 plays a critical role in the functional deterioration of left ventricles during the transition from compensatory hypertrophy to congestive heart failure in salt-sensitive hypertensive rats, Circulation 98 (1998) 2065–2073, https://doi.org/10.1161/01.cir.98.19.2065.
[24] R.B. Sterzel, F.C. Luft, Y. Gao, J. Schnermann, J.P. Briggs, D. Ganten, R. Waldherr, E. Schnabel, W. Kriz, Renal disease and the development of hypertension in salt-sensitive Dahl rats, Kidney Int. 33 (1988) 1119–1129, https://doi.org/10.1038/ki.1988.120.
[25] T. Hayashi, T. Tsujino, S. Iwata, H. Nonaka, N. Emoto, Y. Yano, S. Otani, Y. Hayashi, H. Itoh, M. Yokoyama, Decreased ornithine decarboxylase activity in the kidneys of Dahl salt-sensitive rats, Hypertens. Res. 25 (2002) 787–795, https://doi.org/10.1291/hypres.25.787.
[26] S. Yamamoto, Y. Hotta, K. Maeda, T. Kataoka, Y. Maeda, T. Hamakawa, et al., High salt loading induces urinary storage dysfunction via upregulation of epithelial sodium channel alpha in the bladder epithelium in Dahl salt-sensitive rats, J. Pharmacol. Sci. 135 (2017) 121–125, https://doi.org/10.1016/j.jphs.2017.10.001.
[27] M. Velasquez Flores, A.H. Mossa, P. Cammisotto, L. Campeau, Bladder overdistension with polyuria in a hypertensive rat model, Neurourol. Urodyn. 37 (2018) 1904–1912, https://doi.org/10.1002/nau.23550.
[28] C. Lee, J.P. Etchegaray, F.R.A. Cagampang, A.S.I. Loudon, S.M. Reppert, Posttranslational mechanisms regulate the mammalian circadian clock, Cell 107 (2001) 855–867, https://doi.org/10.1016/s0092-8674(01)00610-9.