Bladder outlet obstruction disrupts circadian bladder function in mice

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The circadian clock programs daily rhythms and coordinates multiple behavioural processes, including micturition. Partial bladder outlet obstruction (pBOO) in mice produces hyperactive voiding. However, long-term effects of pBOO on bladder function have not been clarified. In this study, we investigated micturition under conditions of impaired circadian bladder function by inducing long-term pBOO by tying the proximal urethra. Micturition behavior was evaluated at 1, 3, 6 and 12 months after surgery. We used automated voided stain on paper method for a precise micturition recording for mice. And quantitative assessment of gene expression was performed at 24 months after pBOO surgery using qRT-PCR procedure. The micturition frequencies in the pBOO group were significantly decreased at 3, 6, and 12 months compared to those at 1 month after operation in the same group (p < 0.05). Body weight of pBOO mice was significantly increased compared to sham operated mice at 12 months. The expression level of mRNA was exhibited a 3.4-fold nominal increased for a 5-HT2B receptor in the pBOO group compared to the sham group. The current study found that long-term pBOO led to disruption of the circadian bladder function (the day/night cycle) in mice, similar to those observed in human as nocturia. This disruption is possible involvement of the gain of body weight and/or serotonergic alteration after pBOO.

Abbreviations
BOO  Bladder outlet obstruction
pBOO  Partial bladder outlet obstruction
LUTS  Lower urinary tract symptoms
aVSOP  Automated voided stain on paper
Mets  Metabolic syndrome
CRF  Corticotropin-releasing factor
HPA  Hypothalamus–pituitary–adrenal
α1ARs  α1-Adrenergic receptors

In men as a result of benign prostatic enlargement bladder outlet obstruction (BOO) is one of the most common causes of lower urinary tract symptoms (LUTS). Partial BOO (pBOO) significantly alters bladder morphology and function. Clinically, benign prostatic hyperplasia, bladder neck sclerosis, urethral valves or urethral strictures can cause mechanical BOO1. Although a correlation between LUTS and BOO or metabolic syndrome are known, to our knowledge, the detailed aetiology of LUTS remains unknown. Some symptoms, e.g. nocturia, remain to treat completely due to unclear mechanisms of LUTS. Several studies have shown that nocturia is a common complaint and (one of) the most frequent events in male LUTS. Nocturnal voiding disrupts sleep, and therefore repeated nocturnal voiding deteriorates the health-related quality of life2. There is very little information about the micturition behaviour as circadian bladder function in animal model.

Previous studies have shown that pBOO induces non-voiding contraction and shortening of inter-contraction interval in urinary bladder in animals3,4. Furthermore, pBOO leads to several morphological and functional changes in afferent pathways as well as in the bladder. To empty partially obstructed-bladder, pBOO produces compensate hypertrophy of detrusor, which causes gain of the thickness and weight of the bladder wall5,6. Although...

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Table 1. Summary of results in the aVSOP (automated voided stain on paper) study. Data are presented as mean ± standard deviation; the 95% confidence intervals are shown in brackets. pBOO partial bladder outlet obstruction. † p < 0.05 vs. sham group at the respective month by two-tailed non-paired Student’s t test. * p < 0.05 vs. 1-month group by two-tailed paired Student’s t test.
pBOO procedure, body weight of pBOO mice were significantly increased compared to sham operated mice (34.5 g vs. 25.3 g in median) (*p < 0.05) (Table 1). And, in pBOO mice, surrounding tissue of bladder adjacent was increased and tissue was adipose.

RT-PCR and bladder weight at 2 years after the BOO creation. At 2 years after the BOO creation, mean weight of urinary bladders were 34.5 ± 3.0 mg (n = 4) and 38.3 ± 14.3 mg (n = 7) in the sham and BOO groups, respectively. The result of expression levels of mRNA for several receptors was shown in Table 2. Any mRNA tended to express higher in BOO rats than in sham rats. The expression levels of them were 5-HT2B recep-

| Receptor | Sham, n = 4 | pBOO, n = 7 |
|----------|-------------|-------------|
|          | Mean | Standard deviation | Mean | Standard deviation |
|          | Positive | Negative | Positive | Negative |
| 5-HT2A   | 1.000 | 0.177 | 0.150 | 1.462 | 0.287 | 0.240 |
| 5-HT2B   | 1.000 | 0.820 | 0.450 | 3.407 | 2.988 | 1.592 |
| 5-HT2C   | 1.000 | 1.085 | 0.520 | 1.699 | 1.340 | 0.749 |
| 5-HT4    | 1.000 | 0.336 | 0.251 | 1.342 | 0.295 | 0.242 |
| 5-HT7    | 1.000 | 0.516 | 0.341 | 1.442 | 0.692 | 0.467 |
| α1A      | 1.000 | 0.245 | 0.197 | 1.666 | 1.039 | 0.640 |
| α1D      | 1.000 | 0.561 | 0.359 | 1.561 | 0.889 | 0.567 |
| β1       | 1.000 | 0.242 | 0.195 | 1.350 | 0.221 | 0.190 |
| β2       | 1.000 | 0.465 | 0.317 | 0.947 | 0.164 | 0.140 |
| β3       | 1.000 | 0.279 | 0.218 | 1.402 | 0.272 | 0.227 |
tor > 5-HT2C receptor > 5-HT2A receptor > α1A adrenoceptor > α1D adrenoceptor > 5-HT4 receptor = 5-HT7 receptor, β1 adrenoceptor, β3 adrenoceptor in a descending order. The expression level of β2 adrenoceptor was no changed.

Discussion
Long-term pBOO impairs the circadian bladder function (the day/night cycle) in mice. In mammals, micturation frequency during the sleep period is lower than that during the awake period. Since murine animals are nocturnal, mice have more frequent rhythms for micturition during the sleep phase than during the day. Previous studies have reported that animals with pBOO showed various changes in the lower urinary tract functions. To the best of our knowledge, the study of Murakami et al.13 is the longest study of pBOO using rat model as 6 months. Our group reported that pBOO leads to several morphological and functional changes, which need certain period4. However, it currently remains unclear whether how long needed remodelling and change the function of lower urinary tract. Therefore, the aim of this study was to determine the effects of 12 months effect of pBOO using mice.

Assessing pattern of micturition in mice is challenging14. Because even in adult mice the urine volume voided per micturition is too small, it was not until development of the automated voided stain on paper method that the mice micturition had been assessed continuously and accurately. The fine procedure was devised to record the micturition of a mouse with free moving including an access to food and water for experiment days15. By this procedure, physiological voiding behaviour, e.g., urine volume and frequency, came to be able to measure finely and easy. Negoro et al. also reported that the establishment of a day-night rhythm of adult mice from neonate. This is a key axis for analysing micturition behaviour since the investigation of such rhythms may be helpful to translate into improvement of paediatric nocturnal enuresis or adult nocturia. A murine cystometrogram indicated that pBOO produces bladder hypertrophy and hyperactive voiding16. The cystometrogram is one of the most helpful procedures to measure functions of a urinary bladder, but it cannot replicate physiological condition anaesthesia may affect bladder capacity and diurnal behaviour17. In humans and rodents, functional bladder capacity and rate of urine production during the sleep period are increased and decreased, respectively. By which, they are split off from waking and their sleeping is protected18. The aVSOP system, which has been developed to mimic a frequency-volume chart at a human clinic, is an excellent translational procedure.

The increase in day time micturition frequency and the decrease in average voided volume in day time were observed from 6 months after surgery. And 12 months after pBOO procedure, body weight was significantly increased compared to sham operated mice. In the current study, 6 months or later after pBOO surgery, during harvest bladder, adjacent soft tissue was increased and tissue was adipose. Asplund reported that the increase body weight induced the increasing number of nocturnal voiding. Although the details of mechanisms are remained to be cleared, frequent nocturnal micturition increases the risk of obesity, as could be related the negative impact on sleep18. Recently, both of male and female suffered from LUTS will be overweight and will have features of the metabolic syndrome (Mets). High-Fat diet attenuates amplitude of clock gene expression19. And, increasing body weight, Leptin, which is produced by adipose tissue, controls the major CNS systems that modulate food intake and energy expenditure20. From these perspectives, increase in the body weight of pBOO mice could disrupts circadian bladder function. However, there is no report about the concept that LUTS induce Mets. In the present study, long-term bothersome of LUTS may raise undesirable psychiatric responses, e.g. anxiety, depressive disorders, and hyperorexia. Corticotropin-releasing factor (CRF), which has been identified as a neuropeptide responsible for initiating many of the endocrine, autonomic and behavioural responses to stress, possibly plays a key role. Interestingly, stress, sleep disturbance, and circadian rhythm disruption reciprocally connect in molecular signaling and anatomic pathways. The hypothalamic–pituitary–adrenal (HPA) axis, which is the main pathway of stress response, is regulated by the circadian rhythm21. Psychological stressors also modulate cortisol levels, which is elevated together with the increment of the stress response by circadian misalignment and sleep restriction. Circadian misalignment induces sleep disruption and alters cortisol production22. Stress, circadian rhythms, and sleep all work each other (Fig. 2).

Stress, sleep disturbance, and circadian disruption interact at many levels to affect metabolic disruption. Although the body weight markedly gained in the pBOO group as compared to the sham group (Table 1), to clarify, this mechanism we have to confirm the amount of adipose tissue and the plasma leptin concentrations. However, it is difficult to quantify the amount of adipose tissue especially mice model. This quantification of Mets in small animals to define should be a future issue.

In the present study, the result of RT-PCR was distinguishingly tended to be high for all receptors, particularly a 5-HT2B receptor, except for β2-adrenoceptor in the pBOO group. In the previous study, ketanserin, 5-HT2A/2C receptor antagonist, attenuates detrusor contraction in vitro7. Recently, it was reported that the 5-HT2B receptor is associated with the serotonin-evoked contraction of detrusor strips which is isolated from pBOO rats after 1-week operation, and the detrusor intensify a 5-HT evoked contraction in pBOO rats as compared to sham rats23. In addition, 5-HT2A and 5-HT2B receptor subtypes contribute the contraction at high and low concentration of serotonin, respectively23. Therefore, the present result of overexpression of 5-HT2B receptor suggests that the detrusor easily contracts at a low concentration of serotonin in the pBOO group as compared to the sham group at 2-year after operation. This mention may indicate that a high contractility needs in an obstructed urinary bladder at the old age (Fig. 3). Although further experiments are essential, the long-term obstruction may transform the bladder contractility from overactive to underactive, particularly after 12 months and more from urinary obstruction.

In the last decade much attention has focused on the role of the sympathetic nervous system and α1-adrenergic receptors (α1-ARs) in the dynamic (smooth muscle contraction) component of pBOO. In regard to changes in α1-AR subtype expression following pBOO, Hampel et al. observed in a rat model of pBOO that expression level

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of the α1d-AR mRNA was three times greater than the sham group after 6 weeks of obstruction24. The present results indicated the same trend but were mild increase. The difference between the previous investigation and the present result possibly shows transforming from high frequency at 6 weeks to eliminated frequency at 12 months.

This study has some limitations. The quantification of Mets in mice is a subject for future analysis. And, the main focus of the present study is the experiment of metabolic cage, and RT-PCR is secondary carried out. Although a mismatch of timing of measurement for levels of mRNA is evident limitation, an interpretation can be understood as the long-term results over 12 months.

This is the first study to demonstrate the chronological change of circadian bladder function and body weight change in long-term (12 months) pBOO mice. The current study has found that pBOO lead to disruption of one of the circadian bladder functions (the day/night cycle) in mice, similar to those observed in human as nocturia.

Methods

Animals and study design. Eight-week-old female C57/BL6 mice were housed in a room maintained at 20–26 °C and 35–75% relative humidity, which in the facility is generally stable at approximately 60%, with an alternating 12 h light (from 7 a.m.)/dark cycle (from 7 p.m.). All mice were allowed free access to food pellets

Figure 2. A scheme of the interaction of stress, sleep disturbance, and circadian rhythm disruption promotes body weight gain. Sleep and circadian rhythm reciprocally modulate the hypothalamus–pituitary–adrenal (HPA) axis in normal conditions; however, once chronic stress provokes, followed by the HPA axis constantly activating. Finally, the control of circadian rhythm goes wrong and the rhythm is disrupted. When these interconnected relationships are dysfunctional, the relations will be led to a perpetual negative spiral that synergistically exacerbates pathophysiologic changes in each other. LUTS lower urinary tract symptoms.

Figure 3. Circadian bladder function in point of the micturition rate for 24 h. Circles show the mean total-micturition for 24 h. Areas of black and white reveal percentages of micturition in night and day time, respectively. 5-HT2B-R 5-Hydroxytryptamine 2B receptor
and tap water. The study was conducted in compliance with the Internal Regulations on Animal Experiments at the Institutional Committee. The proximal urethra was tied as in the procedure for pBOO as previously described. In brief, “The bladder and proximal urethra were exposed through a lower abdominal incision. The proximal urethra was freed from the vaginal wall by careful dissection to avoid injury to the periurethral blood vessels. To create an infravesical obstruction, a metal rod with a diameter of 0.55 mm was placed beside the proximal urethra and a 4-0 silk ligature was tied around the urethra and metal rod. The rod was subsequently removed and the abdominal incision was closed.” Sham surgery was performed. Micturition behaviour was evaluated at 1 month, 3 months, 6 and 12 months after pBOO surgery. Additionally, to explore the mechanism on the changes of micturition, quantitative real-time polymerase chain reaction (qRT-PCR) was conducted at 24 months. Animals were treated in accordance with the National Institutes of Health animal care guidelines. All experiments, including protocols, were approved by Institutional Animal Care and Use Committee of National University Corporation Hokkaido University (No. 16-0103).

**Metabolic cage study.** Each mouse was placed in an individual metabolic cage at 1, 3, 6 and 12 months after pBOO and sham surgeries. We used aVSOP (automated voided stain on paper) method, which is a precise micturition recording system for mice. Urine stains were counted and traced, ranging from 10 to 800 μl. Each void was captured by placing, at the base of the cage, a sheet of paper that was rolled up with a laminated filter and pre-treated to turn the edge of urine stains deep purple. The paper was wound up at a speed of 10 cm/h under a water-repellent wire lattice. Urine stains were counted and traced, and stain sizes were converted to micturition volume by using the formula of a standard curve. The standard curve was generated by dropping normal saline (in volumes of 10–800 μl) onto similarly treated paper and tracing the resulting stained areas. To relieve stress, the mice were provided with free access to water and food while housed in the individual metabolic cages. To reduce voiding activity that might result from any stress, the animals were habituated to the metabolic cages for 24 h before initiating measurement of the voiding events. Mice were placed into individual metabolic cages for 24 h and voids were captured with paper at the base of the cage. The parameters evaluated were voided volume and time per void, total urination frequency (daytime and night time) and total urine volume.

**Tissue homogenization.** A bladder tissue was cut into several specimens of 20 mg or less. Individually, the specimen was each transferred into a 2 mL-tube containing 4.8 mm of a bead made from stainless steel and a lysis buffer. Homogenization was performed in the tube for ten runs of 30 s each at 3,500 rpm using Micro smash™ (MS-100R; TOMY SEIKO, Tokyo, Japan). The tube was cooled at 4 °C for 30 s between the runs.

**Quantitative RT-PCR.** Quantitative assessment of gene expression was performed using qRT-PCR according to the manufacturer’s manual. RNA was extracted from the urinary bladder using a RNeasy Fibrous Tissue Mini Kit (#74704; QIAGEN, Venlo, Netherlands). Purified RNA was reversely transcribed cDNA using a SuperScript VILO cDNA Synthesis Kit (#11754-050; Invitrogen, Massachusetts, USA). The experimental procedure of each kit was carried out according to the manufacturer’s instructions. Quantitative real-time polymerase chain reaction was performed using Quant Studio 7 Flex (#4484643; Applied Biosystems, Massachusetts, USA) with Taqman (Applied Biosystems, Massachusetts, USA) and THUNDERBIRD (#QPS-101; TOYOBO, Osaka, Japan). RPL 19 was applied as housekeeping gene. Relative expression level of each mRNA was then normalized to the expression level of RPL 19, using the 2−ΔΔCT method.

**Statistical analysis.** Statistical analyses were conducted with the GraphPad Prism for Windows (GraphPad Software, San Diego, CA, USA). All data are expressed as mean ± standard deviation. Student’s t test was used to compare the metabolic cage parameters pBOO mice to sham operated mice. Dunnett’s test was performed to compare the values at each month after pBOO surgery with referring to the value at 1 month. P values <0.05 were statistically significant. Regarding qRT-PCR, the mean of the pBOO group is shown as ratio referred to the sham group.

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Competing interests
The authors declare no competing interests.

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