

## ORIGINAL ARTICLE

# Presence of multiple peripheral circadian oscillators in the tissues controlling voiding function in mice

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Circadian clocks are the endogenous oscillators that harmonize a variety of physiological processes within the body. Although many urinary functions exhibit clear daily or circadian variation in diurnal humans and nocturnal rodents, the precise mechanisms of these variations are as yet unclear. In the present study, we demonstrate that *Per2* promoter activity clearly oscillates in neonate and adult bladders cultured *ex vivo* from *Per2::Luc* knock-in mice. In subsequent experiments, we show that multiple local oscillators are operating in all the bladder tissues (detrusor, sphincter and urothelium) and the lumbar spinal cord (L4–5) but not in the pontine micturition center or the ventrolateral periaqueductal gray of the brain. Accordingly, the water intake and urine volume exhibited daily and circadian variations in young adult wild-type mice but not in *Per1*<sup>-/-</sup>*Per2*<sup>-/-</sup> mice, suggesting a functional clock-dependent nature of the micturition rhythm. Particularly in PDK mice, the water intake and urinary excretion displayed an arrhythmic pattern under constant darkness, and the amount of water consumed and excreted significantly increased compared with those of WT mice. These results suggest that local circadian clocks reside in three types of bladder tissue and the lumbar spinal cord and may have important roles in the circadian control of micturition function. *Experimental & Molecular Medicine* (2014) 46, e81; doi:10.1038/emm.2013.153; published online 7 March 2014

**Keywords:** bladder; circadian clock; lumbar spinal cord; peripheral oscillator; voiding; water intake

## INTRODUCTION

Organisms on this rotating planet have adaptations for periodic changes in environmental conditions, such as environmental illumination, temperature, humidity, and food and predator availability. Actually, almost every life form on Earth displays obvious daily or circadian variations in biochemistry, physiology, pathology and behavior.<sup>1–5</sup> In mammals, the suprachiasmatic nucleus of the hypothalamus acts as a master clock and orchestrates the peripheral clocks in various tissues and organs. Thus, virtually all organisms on Earth have successfully developed endogenous mechanisms that harmonize a variety of behavioral and physiological processes according to the time of day.<sup>2,4,5</sup> With an effective circadian timing system, most physiological functions in mammals are tightly locked to their activity and rest phases to ensure optimal performance.

In accordance, distinct daily variations in urinary function and behavior are obvious in diurnal humans and nocturnal

rodents.<sup>6–8</sup> Intuitively, most urine production and voiding should occur during the active phase, whereas better rest and sleep require increased storage of urine in the bladder and reduced urination frequency. Disruptions of proper voiding rhythms are predicted to negatively affect the organism's wellbeing. For example, nocturia, abnormal waking during the night one or more times to void,<sup>9–11</sup> is known to decrease quality of life and negatively affects morbidity and mortality, especially in the elderly.<sup>12–15</sup> Possible causes of nocturia have been attributed to bladder storage problems, diurnal polyuria and nocturnal polyuria,<sup>10</sup> but the presence and functionality of local peripheral clocks in the regions controlling voiding functions have not yet been fully established. Recently, Herrera and Meredith (2010) observed day vs night differences in bladder capacity and voiding frequency in rats, raising the possibility that bladder function can be a direct target of circadian regulation.<sup>16</sup> As nocturia is one of the most common urinary storage symptoms in most developed and

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developing countries,<sup>17,18</sup> a clearer understanding of voiding function and dysfunction is necessary. The possible existence of local peripheral clocks in the tissues controlling micturition function has yet to be determined.

In the present study, we demonstrate operating circadian oscillations in the lumbar spinal cord as well as in three distinct tissues of the bladder. Moreover, we provide evidence that circadian variations in micturition function are dependent on a functional molecular clock.

## MATERIALS AND METHODS

### Animals, entrainment and circadian sampling

All animal experiments were approved by and performed under the guidelines of Kyung Hee University Institutional Animal Care and Use Committee. The *Per1*<sup>-/-</sup>*Per2*<sup>-/-</sup> (PDK)<sup>19</sup> and *Per2::Luc* knock-in<sup>20</sup> mice were described previously. All mice were raised in a light-proof Clean Animal Rack cabinet (Shin Biotech, Seoul, Korea) with constant air ventilation, and these cabinets were maintained in a temperature-controlled room (23 ± 1 °C). The light intensity during the light phase was kept at 350–450 lux throughout the area. Young adult WT, PDK and *Per2::Luc* knock-in mice were entrained to a 12-h light/12-h dark (LD, light/dark 12:12) photoperiodic cycle with lights on at 0800 hours. After 1 or 2 weeks of entrainment, the mice were kept in constant darkness (DD). On the second day after the lights were turned off, the animals were sacrificed by injection of chloral hydrate (360 mg kg<sup>-1</sup>, intraperitoneal) at the indicated circadian time.

### Real-time monitoring of *Per2* promoter activity from bladder and spinal cord

Adult *Per2::Luc* knock-in mice were anesthetized with chloral hydrate (360 mg kg<sup>-1</sup>, intraperitoneal). Whole bladder samples, dissected bladder tissues or spinal cord fragments (lumbar 4–5) were used for real-time monitoring of *Per2* promoter activity, depending on the experiment. For the whole bladder samples, the bladder outlet was tied using thread, the remaining urine was removed using a syringe, and the bladder was filled with MEM (Gibco, Carlsbad, CA, USA) supplemented with 25% of Gey's balanced salt solution, 25% fetal bovine serum, 100 units ml<sup>-1</sup> penicillin, 100 µg ml<sup>-1</sup> streptomycin, 36 mM glucose and 0.1 mM luciferin (Promega, Madison, WI, USA). The whole bladder samples were immobilized in a 35-mm culture dish (Corning, Tewksbury, MA, USA) using cell strainers (SPL, Seoul, Korea), and the bioluminescence was continuously recorded using a KRONOS apparatus (ATTO, Tokyo, Japan). To monitor neonate bladders, bladders were obtained from newborn mice, and tissues were sliced with a blade. The tissue slices were immobilized in a culture insert and incubated for 1 day after a medium change. Then, the tissue slices were synchronized by a dexamethasone shock for 3 h. These tissues were monitored using the same procedure as for the adult bladder tissues. For the bladder tissue samples and the spinal cord, samples were obtained under a dissecting microscope and cultured separately on Millicell culture membrane inserts (Millipore, Billerica, MA, USA) with 1 ml of MEM, supplemented with 25% of Gey's balanced salt solution, 25% fetal bovine serum, 100 units ml<sup>-1</sup> penicillin, 100 µg ml<sup>-1</sup> streptomycin and 36 mM glucose. The samples were then transferred to 35-mm culture dishes with recording medium. The bioluminescence was continuously recorded in the same manner as for the whole bladder.

### RNA isolation, reverse transcription and real-time PCR

Total RNA was extracted from the bladder (urothelium, detrusor smooth muscle or sphincter smooth muscle), spinal cord (lumbar 4–5), pontine micturition center (PMC) and ventrolateral periaqueductal gray (vlPAG) fragments using the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method as described previously.<sup>21</sup> The RNA concentration was determined using an ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). One µg of total RNA and 200 ng of random hexamers (Takara, Shiga, Japan, 3801) in a total volume of 11 µl were incubated for 5 min at 65 °C and chilled on ice. Then, 4 µl of 5X Reverse transcriptase buffer, 4 µl of 2.5 mM each of dNTPs, 0.5 µl of RNase inhibitor (40 units, Takara, 2310A) and 0.5 µl of Reverse transcriptase M-MLV (200 units, Takara, 2640 A) were added, and the reaction mixture was incubated for 1 h at 37 °C and for 10 min at 70 °C. The procedure for real-time RT-PCR using LightCycler has been described previously.<sup>22</sup> The standards were prepared by pooling fivefold diluted cDNA samples in 1 mM Tris. The 5-fold diluted cDNA samples were further diluted 15-fold to prepare templates for real-time RT-PCR. Real-time RT-PCR was performed on a LightCycler Version 1.5 (Roche, Indianapolis, IN, USA) using 2X SYBR Premix EX Taq (Takara, RR041A). The expression levels of *Gapdh* were used for normalization. The following primer sequences were used for real-time RT-PCR: *Bmal1* up (upregulated), 5'-GGCC ATCAGTAAAGGTGGAA-3'; *Bmal1* dn (downregulated), 5'-GGTGG CCAGCTTTTCAAATA-3'; *Rev-erba* up, 5'-AAGACATGACGACCTG GAC-3'; *Rev-erba* dn, 5'-GAGTCAGGGACTGGAAGCTG-3'; *Per1* up, 5'-GTGTCGTGATTAATAGTACAG-3'; *Per1* dn, 5'-ACCACTCATG TCGTCTGGGCC-3'; *Cry1* up, 5'-CGAATGAATGCAAACCTCCCT-3'; *Cry1* dn, 5'-AAAAATTCACGCCACAGGAG-3'; *Gapdh* up, 5'-AAC TTTGGCATTGTGGAAGG-3'; and *Gapdh* dn, 5'-ACACATTGGGGG TAGGAACA-3'.

### Measurement of water intake and urine excretion

To determine the water intake and urine excretion, WT and PDK mice (8–10 weeks) were housed individually in metabolic cages (Jeungdo, Seoul, Korea). These animals were entrained to a 12-h light/12-h dark (LD 12:12) photoperiodic cycle with lights on at 0800 hours for a week. On the 8th day, the water intake and urine excretion were measured at 2 h intervals throughout a day. Then, the lights were turned off on the 9th day. On the second day after the lights were turned off, the water intake and the urine excretion were determined every 2 h in the DD condition throughout a circadian cycle.

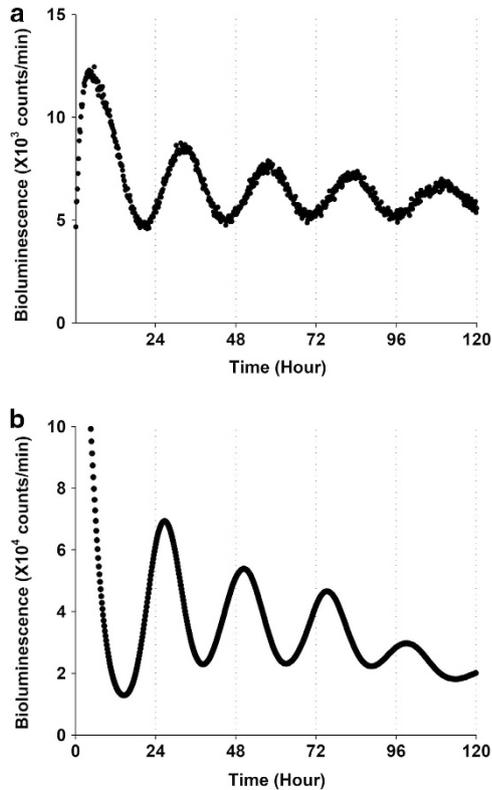
### Statistical analysis

Water intake and urine excretion were statistically analyzed by Student's *t*-test for comparison between the two groups. Statistical significance was set at *P* < 0.05.

## RESULTS

### Rhythmic oscillation of *Per2* promoter activity in the whole bladders of neonate and adult mice

To verify the existence of an autonomous circadian clock in the isolated organotypic whole bladder, we utilized *Per2::Luc* knock-in mice<sup>20,23</sup> because these animals allow us to detect the oscillation in the transcription of a canonical clock gene, *Per2*, in whole organs or tissues quite easily. As shown in Figures 1a and b, organotypic *ex vivo* cultures of the whole bladder from adult as well as neonate mice displayed rhythmic oscillations of the *Per2* promoter activity with a free-running



**Figure 1** Representative recordings of the bioluminescence from the whole bladders of *Per2::Luc* mice. Whole bladders from neonate (a) and young adult (b) *Per2::Luc* mice were analyzed for *Per2* promoter-driven luciferase activity. (a) Adult *Per2::Luc* knock-in mice were anesthetized with chloral hydrate (360 mg kg<sup>-1</sup>, intraperitoneal), and the whole bladders were retrieved. Then, the bladder outlet was tied using thread, the remaining urine was removed using a syringe, and the bladder was filled with MEM (Gibco) supplemented with the appropriate medium. The whole bladder samples were immobilized in a 35-mm culture dish (Corning, Tewksbury, MA, USA) using cell strainers (SPL, Seoul, Korea), and the bioluminescence was continuously recorded using a KRONOS apparatus (ATTO, Tokyo, Japan). (b) To monitor neonate bladders, bladders were obtained from newborn mice, and the tissues were sliced using a blade. The tissue slices were immobilized in a culture insert and incubated for 1 day after a medium change. Then, the tissue slice were synchronized by dexamethasone shock for 3 h. Monitoring was performed using the same method as for the adult bladder tissues. The bioluminescence was continuously recorded for more than 5 days using a KRONOS apparatus as described in *Methods*.

period of ~24 h. The oscillation of *Per2* promoter activity in the isolated whole bladders indicates that the *Per2* promoter is active in the bladder of neonate and adult mice and that the rhythmic transcription of *Per2* gene is intrinsic to a bladder that is operating without any cues from other body parts.

#### Presence of functional peripheral clocks in three distinct tissues of the bladder

The urinary bladder can be divided into three functional parts: detrusor smooth muscle, sphincter smooth muscle, and urothelium.<sup>24,25</sup> Once we confirmed the intrinsic oscillations

of *Per2* promoter activity, our next question was whether circadian clocks operate in the three distinct functional tissues of the bladder. To this end, we rapidly obtained these tissues under a dissecting microscope and continuously recorded the resulting bioluminescence for 5 days using a KRONOS apparatus. In addition, we examined the expression patterns of other clock genes (*Per1*, *Bmal1* and *Rev-erb $\alpha$* ) over a circadian cycle in DD by quantitative real-time RT-PCR. As shown in Figure 2, all the functional parts of the bladder exhibited distinct and persistent oscillations in *Per2* bioluminescence with a period of ~24 h. The periods and acrophases were indistinguishable among the three types of tissue. Moreover, all canonical clock genes examined in this study oscillated in the wild-type mice but not in the arrhythmic *Per1*<sup>-/-</sup> *Per2*<sup>-/-</sup> (*Pers* double knockout; PDK) mice. The persistent oscillation of circadian clock genes in separated bladder tissues indicates the intrinsic nature of local peripheral clocks in the three functional tissues of the bladder.

#### Functional clockwork also operates in the lumbar spinal cord of mice

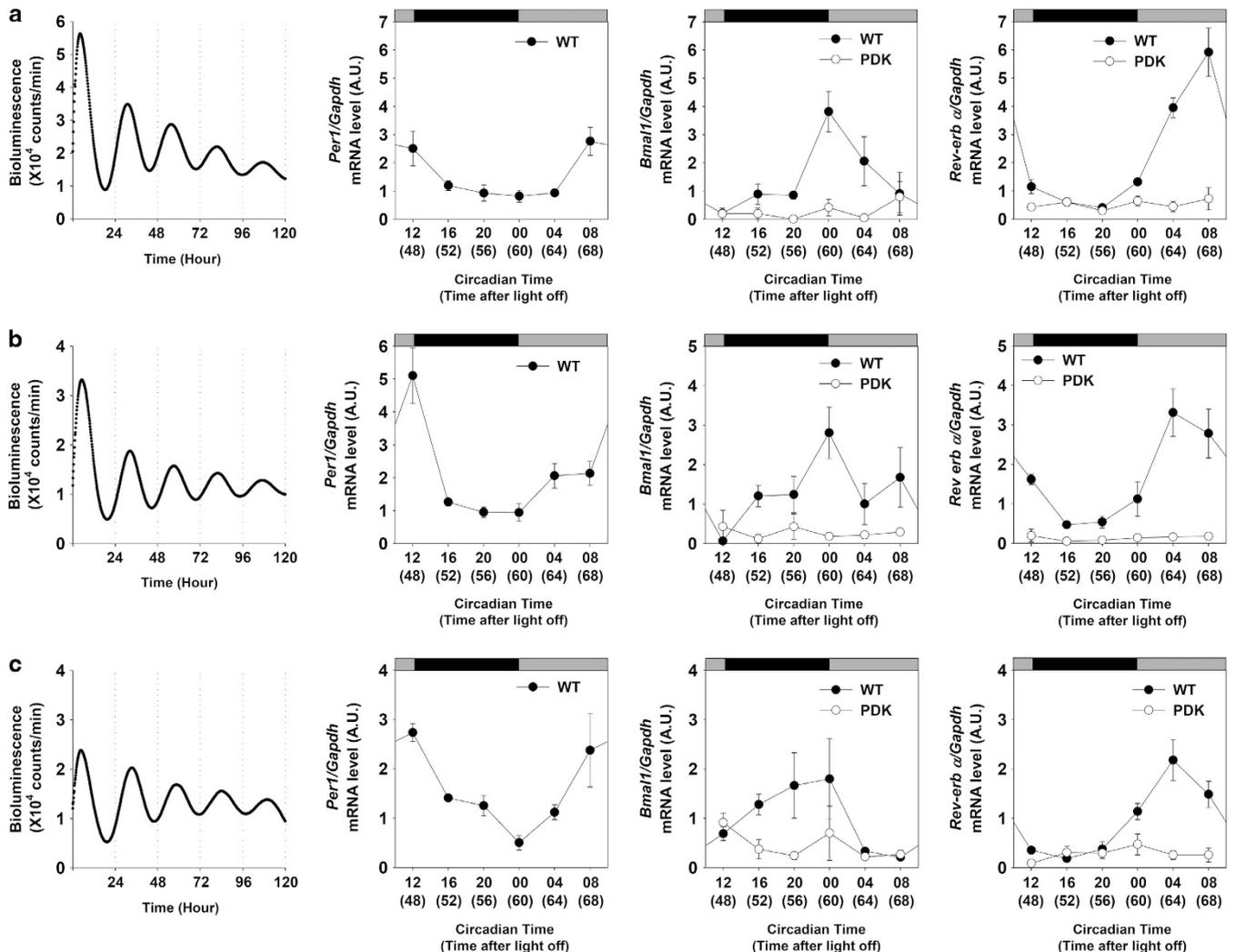
The control of voiding functions by central nervous system inputs is well known. In particular, the lumbar spinal cord has been connected to voiding and urine storage reflexes.<sup>25</sup> Thus, we investigated whether functional circadian clockwork also exists in this part of the spinal cord, that is, lumbar 4–5 region. We first examined the *Per2* promoter activity in the isolated lumbar spinal cords from *Per2::Luc* mice (Figure 3; upper left), which revealed a persistent circadian oscillation in the bioluminescence. In addition, the expression of other clock genes was analyzed by real-time RT-PCR, which demonstrated that *Per1*, *Bmal1* and *Rev-erb $\alpha$*  oscillated in the lumbar spinal cord in the WT mice in the absence of an exogenous LD cycle (Figure 3); however, the amplitudes were smaller than those of the bladder tissues (compare with Figure 2). As expected, the PDK mice did not exhibit any significant oscillation. These results indicate the presence of functional circadian clockwork in the lumbar spinal cord, which operates intrinsically without cues from other body parts.

#### No oscillations of clock genes in the PMC and vPAG

Higher brain regions, such as the PMC and vPAG, are also involved in the neural control of micturition function.<sup>24–27</sup> Thus, we investigated whether any functional circadian clockworks exist in these regions of the brain (Figure 4). Although the expression of clock genes was evident in these regions, no clear oscillation was detected by quantitative real-time RT-PCR. Moreover, the *Rev-erb $\alpha$*  expression levels of the WT mice were similar to those of PDK mice, indicating the absence of a functional peripheral clock in the PMC and vPAG.

#### Daily patterns of water intake and urine volume in WT and PDK mice: effect of an exogenous LD cycle

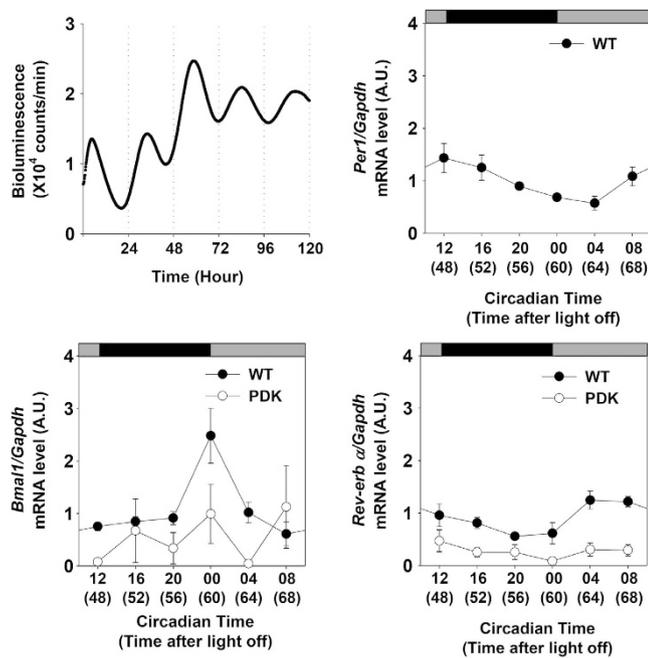
After establishing the existence of peripheral circadian clockworks in some of the voiding-related tissues, our next focus



**Figure 2** Existence of functional circadian clockworks in three distinct tissues of the bladder. (Far left panels of **a–c**) Detrusor smooth muscle (**a**), sphincter smooth muscle (**b**), and uroepithelial layer (**c**) of adult *Per2::Luc* mouse bladders were obtained under a dissecting microscope and analyzed for *Per2* promoter-driven luciferase activity. The bioluminescence was continuously recorded for more than 5 days using a KRONOS apparatus as described in Methods. Representative recordings of bioluminescence are shown here. (Next three panels of **a–c**) Expression of clock genes in three bladder tissues of WT and PDK mice. WT and PDK mice were entrained to a 12L:12D photoperiodic cycle for a week and then kept in constant darkness (DD). Two days after the lights were turned off, the mice were sacrificed by cervical dislocation at the indicated circadian time, and the detrusor smooth muscle (**a**), sphincter smooth muscle (**b**) and uroepithelial layer (**c**) were quickly obtained under a dissecting microscope. Real-time RT-PCR was performed to determine the mRNA levels of *Per1*, *Bmal1* and *Rev-erb $\alpha$* . All mRNA levels were normalized to the *Gapdh* mRNA level and expressed as the mean  $\pm$  s.e.m. ( $n=4$ ). L, light; D, dark; PDK, double knockout of *Per1* and *Per2* genes, WT, wild type.

was determining their functional significance. Thus, we examined water intake and voiding volume not only in WT mice but also in arrhythmic PDK mice. To establish daily patterns of water intake and urine excretion, males of both genotypes were individually housed in metabolic cages and entrained to a 12L:12D photoperiodic cycle for a week. Then, their water intake and urine volume were simultaneously determined every 2 h throughout the eighth day (Figure 5a). In the presence of an LD cycle, the WT male mice exhibited daily variations in water intake and urine volume with an increased amount of water consumed and excreted during the active/

dark phase. Furthermore, daily variations in water intake and urine volume were evident in the PDK male mice, although the day vs night difference in urine volume was statistically insignificant. This observed day vs night difference in PDK mice is consistent with the seemingly rhythmic locomotor activity observed in the presence of an exogenous LD cycle.<sup>28,29</sup> Interestingly, the PDK mice consumed more water than did the WT mice at the time of the light-dark transition and showed a subsequent increase in urine volume 2–4 h after the increased water consumption, suggesting that water intake and urine volume in PDK mice are largely affected by the presence



**Figure 3** A functional circadian oscillator in the lumbar spinal cord. (Upper left) A representative recording of the bioluminescence from the lumbar spinal cord of *Per2::Luc* mice. The bioluminescence was measured from cultures of spinal cord fragments (L4–5) for more than 5 days, as in Figure 1. (Next three panels) WT and PDK mice were entrained to a 12L:12D photoperiodic cycle for a week and then kept in constant darkness (DD). Two days after the lights were turned off, the mice were sacrificed by cervical dislocation at the indicated circadian time, and the spinal cord (lumbar 4–5 region) was quickly obtained. Real-time RT-PCR was performed to determine mRNA levels of *Per1*, *Bmal1* and *Rev-erb $\alpha$* . All mRNA levels were normalized to the *Gapdh* mRNA level and expressed as the mean  $\pm$  s.e.m. ( $n=4$ ). L, light; D, dark; PDK, double knockout of *Per1* and *Per2* genes, WT, wild type.

of exogenous light. Moreover, the 24-h water intake and urine volume of the PDK mice were higher than those of the WT in most cases, suggesting polydipsia and polyuria in these mutant animals.

### Circadian patterns of water intake and urine volume in WT and PDK mice: intrinsic nature of water consumption and excretion rhythms

To determine whether the apparent rhythmic pattern observed in the PDK mice under the LD condition is due to the masking effect of exogenous light, the lights were turned off on the 9th day. On the second day after the lights were turned off, the water intake and the urine volume were simultaneously determined every 2 h throughout a circadian cycle under the DD condition (Figure 5b). Even in the DD, the WT mice still exhibited significant variations in water intake and urine volume, showing a subjective night vs subjective day difference. In contrast, the PDK mice quickly lost their apparent rhythms of water intake and excretion under the same

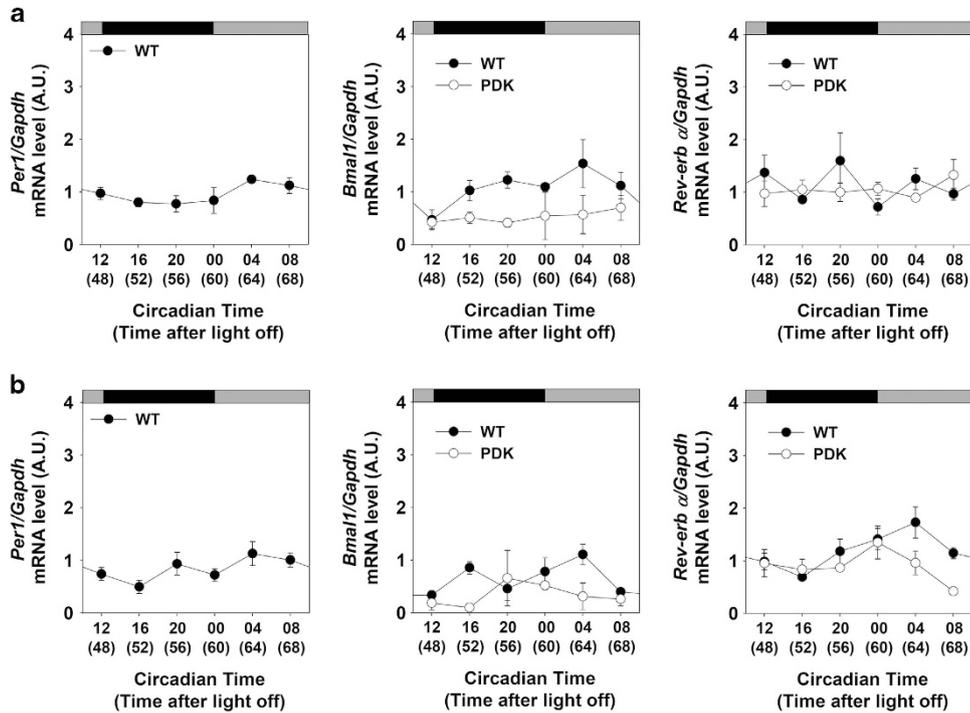
condition. The complete loss of rhythmicity in the PDK mice suggests an endogenous clock-dependent nature of water consumption and urinary excretion in WT mice. The time at which the minimal volumes of urine were collected is particularly notable. Indeed, the urine volumes reached the minimum 3–4 h before the light-dark transition in the WT mice (Figure 5a), and this minimum was maintained in the WT mice even in the absence of an exogenous lighting cue (Figure 5b). Thus, we can conclude that the urine volume in WT mice is dependent on the functional clock but not on the exogenous light or the locomotor activity level.

## DISCUSSION

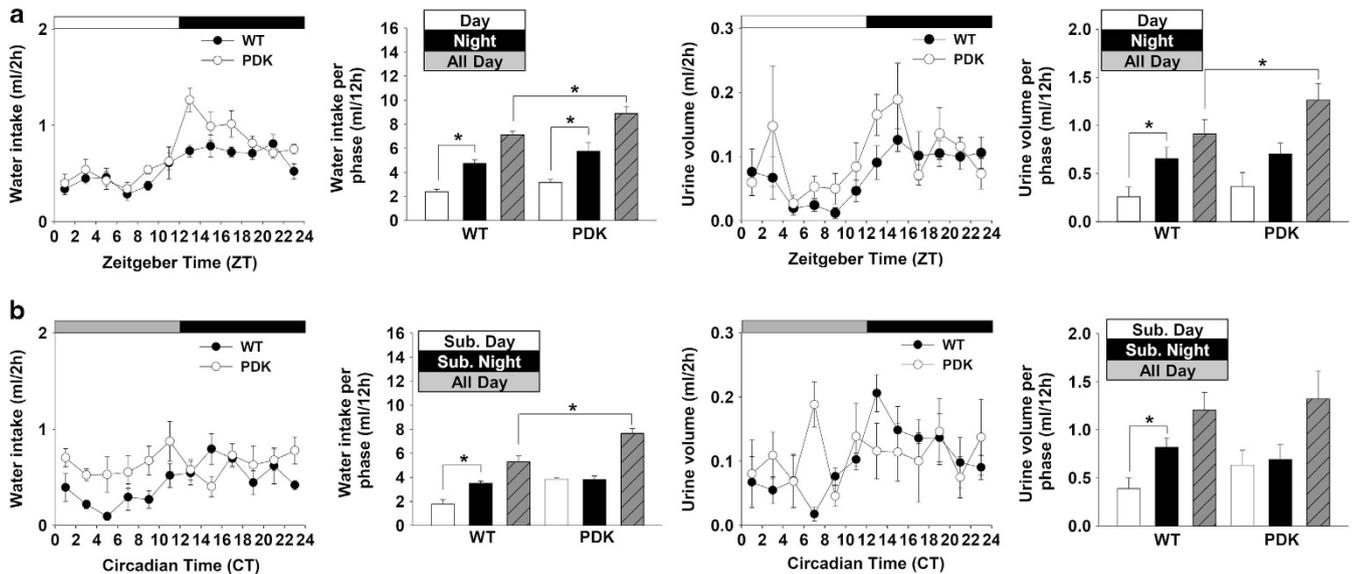
Extensive studies have demonstrated diurnal or circadian variations in urine volume, electrolyte excretion, uroflow, voiding frequency, volume per void, and urine and osmole output rates.<sup>7,30–34</sup> Moreover, arginine vasopressin synthesized in the magnocellular neurons of the hypothalamus displays distinct diurnal or circadian variations and is implicated in many urological problems.<sup>35–37</sup> However, the presence of circadian oscillators in the local regions that control voiding function has so far been largely unnoticed. In the present work, we demonstrated that local circadian clockworks operate intrinsically in the bladder and lumbar spinal cord, the local regions that control voiding function in mice, even in the absence of extrinsic time cues. We also provided evidence that water intake and urinary excretion are controlled in a functional clock-dependent manner, with a possible indication of polydipsia and polyuria in clock-defective PDK mice.

The present work clearly demonstrated persistent oscillation of clock genes not only in the whole bladder but also in three isolated bladder tissues (detrusor, sphincter and urothelium) and lumbar spinal cord fragments (L4–5); the oscillation periods of  $\sim 24$  h were similar. However, we failed to detect any significant oscillation in circadian genes in the PMC and vIPAG, the higher brain regions that control micturition function; therefore, the clock ticks only in some but not all micturition function-related tissues. Moreover, the amplitudes of the circadian oscillation were much higher in the bladder tissues than in the lumbar spinal cord, suggesting that the clock ticks more robustly in peripheral tissues. Although the oscillation of *Per2* transcription in the bladder slice cultures has very recently been reported,<sup>38</sup> this work is the first to demonstrate the presence of multiple peripheral oscillators in micturition function-related tissues. Detrusor smooth muscle, sphincter smooth muscle, urothelium and lumbar spinal cord have distinct roles in the control of micturition and can be separately targeted by various drugs;<sup>24,25,27,39</sup> therefore, the roles of these peripheral oscillators must be resolved in future work.

In this study, the functional clock-defective PDK mice displayed daily variations in water intake and urine volume in the presence of an exogenous LD cycle (Figure 5a), whereas this apparent rhythmicity disappeared just 2 days after the mice were kept in DD (Figure 5b). Thus, the seemingly normal



**Figure 4** No evident rhythms in the expression clock genes in the PMC and vPAG. WT and PDK mice were entrained to a 12L:12D photoperiodic cycle for a week and then kept constant darkness (DD). Two days after the lights were turned off, mice were sacrificed by cervical dislocation at the indicated circadian time. The pontine micturition center (PMC, **a**) and ventrolateral periaqueductal gray (vPAG, **b**) were quickly obtained under a dissecting microscope. Real-time RT-PCR was performed to determine the mRNA levels of *Per1*, *Bmal1*, and *Rev-erbα*. All mRNA levels were normalized to the *Gapdh* mRNA level and expressed as the mean  $\pm$  s.e.m. ( $n=4$ ). L, light; D, dark; PDK, double knockout of *Per1* and *Per2* genes, WT, wild type.



**Figure 5** Daily and circadian patterns of water intake and urine excretion in WT and PDK mice. **(a)** Daily patterns of water intake and urine excretion. WT and PDK mice were housed individually in metabolic cages. These animals were further entrained to a 12-h light/12-h dark (LD 12:12) photoperiodic cycle with lights on at 0800 hours for a week. On the 8th day, the water intake and urine excretion were determined at 2-h intervals throughout a day and are shown as line graphs. Then, the 12-h day, 12-h night and 24-h all-day amounts were pooled and are shown as bar graphs on the right side. **(b)** Circadian patterns of water intake and urine excretion. To determine the circadian patterns, the lights were turned off on the 9th day. Two days after the lights were turned off, the water intake and urine excretion under the DD condition were determined at 2-h intervals throughout a circadian cycle and are shown as line graphs. Then, the 12-h subjective day, 12-h subjective night and 24-h all-day amounts were pooled and are shown as bar graphs on the right side (**b**). \* $P<0.05$ . LD, light/dark; DD, constant darkness; PDK, double knockout of *Per1* and *Per2* genes, WT, wild type.

rhythmicity of the PDK mice under the LD condition can be simply explained by the masking influences of the environmental light. In other words, when environmental light inhibits locomotor activity in rodents,<sup>28,29</sup> the light-induced changes in the activity level in PDK mice lead to alterations in water intake and subsequently changes the urine volume. In support of this notion, the PDK mice consumed the highest amount of water especially at the time of the light-dark transition (Figure 5a) when their locomotor activity also intensifies.<sup>29</sup> The involvement of external time cues as well as the central rhythm that is conveyed to the voiding organs presumably via neural inputs in these processes cannot, however, be ruled out. Thus, the statistical significance was greater for the LD condition than for DD condition. Two-way analysis of variance showed statistical significance for LD depended on the genotype ( $P < 0.001$ ) and ZT ( $P < 0.001$ ) but not for their interaction ( $P > 0.05$ ), whereas under the DD condition, statistical significance was obtained only for the genotypes ( $P < 0.001$ ).

We also observed that the water intake and urine excretion substantially increased in the PDK mice compared with those in the WT mice, possibly indicating polydipsia and polyuria in the PDK mice. Recently, Zuber *et al.* (2009) reported that *Clock*<sup>-/-</sup> mice exhibit diverse phenotypes, including partial diabetes insipidus.<sup>40</sup> Indeed, disturbances of circadian rhythms or knockouts of circadian clock genes have been linked to various pathologies, including mental illness, cancer, obesity, diabetes, cardiovascular disease and aging.<sup>4,41</sup> As discussed previously,<sup>6-8</sup> most urine production and voiding should occur during the active phase, whereas better rest and sleep require increased storage of urine in the bladder and reduced voiding frequency. Thus, disruption of the endogenous clockwork in PDK mice may result in urinary problems, such as polyuria. However, the exact mechanism must be clarified in future works. In this respect, a recent report by Negoro *et al.* (2012) showing the circadian oscillation of *connexin43* in the control of bladder capacity provides a good exemplar.<sup>38</sup> However, more extensive studies are required to address other possibilities. In particular, mouse models carrying tissue-specific disruptions of molecular clockwork are needed to address the tissue-specific roles of local circadian oscillators in voiding function and dysfunction. Because functional circadian clockwork exists in three distinct bladder tissues and the lumbar spinal cord but not in the PMC and vLPAG, tissue-specific ablation of circadian clocks may reveal tissue-specific functions of the local peripheral clocks.

In conclusion, we demonstrated the presence of intrinsically operating molecular clockwork in all the bladder tissues and the lumbar spinal cord. The present work opens a new research avenue for studying the possible roles of peripheral clocks in voiding function and dysfunction.

## ACKNOWLEDGEMENTS

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