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The circadian clock is disrupted in mice with adenine-induced tubulointerstitial nephropathy

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Chronic Kidney Disease (CKD) is increasing in incidence and has become a worldwide health problem. Sleep disorders are prevalent in patients with CKD raising the possibility that these patients have a disorganized circadian timing system. Here, we examined the effect of adenine-induced tubulointerstitial nephropathy on the circadian system in mice. Compared to controls, adenine-treated mice showed serum biochemistry evidence of CKD as well as increased kidney expression of inflammation and fibrosis markers. Mice with CKD exhibited fragmented sleep behavior and locomotor activity, with lower degrees of cage activity compared to mice without CKD. On a molecular level, mice with CKD exhibited low amplitude rhythms in their central circadian clock as measured by bioluminescence in slices of the suprachiasmatic nucleus of PERIOD 2::LUCIFERASE mice. Whole animal imaging indicated that adenine treated mice also exhibited dampened oscillations in intact kidney, liver, and submandibular gland. Consistently, dampened circadian oscillations were observed in several circadian clock genes and clock-controlled genes in the kidney of the mice with CKD. Finally, mice with a genetically disrupted circadian clock (*Clock* mutants) were treated with adenine and compared to wild type control mice. The treatment evoked worse kidney damage as indicated by higher deposition of gelatinases (matrix metalloproteinase-2 and 9) and adenine metabolites in the kidney. Adenine also caused non-dipping hypertension and lower heart rate. Thus, our data indicate that central and peripheral circadian clocks are disrupted in the adenine-treated mice, and suggest that the disruption of the circadian clock accelerates CKD progression.

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Translational Statement

Sleep disorders are prevalent in chronic kidney disease patients, although the underlying mechanisms are not understood. The current study demonstrates that adenine-induced tubulointerstitial nephropathy disrupted the circadian system both centrally and in peripheral organs. *Clock* mutant mice were also more vulnerable to the effects of adenine. These findings aid the understanding of sleep disturbances in adenine phosphoribosyltransferase deficiency, a rare inherited metabolic disorder that leads to the accumulation of 2,8-dihydroxyadenine. More broadly, the results suggest that circadian disruption caused by environmental factors such as nighttime shift work may be a risk factor for chronic kidney disease development.

The number of dialysis patients has been increasing around the world, and chronic kidney disease (CKD) has become a global health issue.[1] Currently, there is no cure for CKD, and treatment options, including kidney transplantation or a lifetime of dialysis, are limited. Further, CKD patients have a high risk of complications, including stroke,[2] cardiovascular disease,[3] fractures,[4] and sarcopenia.[5] Therefore, it is important to investigate approaches to reduce the risk of CKD early in the disease progression. Several studies have reported that CKD patients show fragmentation of sleep, shorter sleep duration, poor sleep quality, and difficulties in the timing of sleep.[6] More than 50% of CKD patients suffer from daytime sleepiness.[7] Clinical reports show abnormal electroencephalogram patterns in CKD patients during wakefulness, especially at the later stages of the disease,[8] and a positive correlation between the abnormal electroencephalogram and serum (blood) urea nitrogen (BUN) has been reported.[9] These studies raise the possibility

that the circadian timing system may be disrupted in CKD patients.

The circadian system controls daily fluctuation of physiological functions in mammals. The central clock, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, regulates the peripheral clocks located in each organ to generate physiological rhythms.[10–12] In the cellular circadian clocks, CLOCK/BMAL1 works as a transcriptional activator to initiate transcription of the *Per1/2/3* and *Cry1/2* genes, and the PER/CRY complex inhibits transcriptional activity of CLOCK/BMAL1 on a cycle approximating 24 hours. Retinoic acid–related orphan receptor (ROR) and REV-ERBα/β activate and suppress *Bmal1* transcription, respectively, to augment the 24-hour rhythm. Clock-controlled PAR-domain basic leucine zipper transcription factors albumin D-box binding protein (DBP), thyrotroph embryonic factor (TEF), and hepatic leukemia factor (HLF) are highly expressed in the kidney with circadian rhythmicity, and they regulate renal functional genes, such as key regulators of water and sodium balance including the vasopressin V2 receptor (*V2r*), aquaporin-2 (*Aqp2*), *Aqp4*, and endothelial sodium channel α (αENaC).[13] It is well known that renal functions, including homeostatic control of water, electrolyte balance, and erythropoietin levels, show circadian rhythmicity.[14] Mice with a genetically disrupted circadian timing system (e.g., *Clock* mutant mice) exhibit both nocturia and nocturnal polyuria with lower expressions of several renal genes.[15] These studies suggest that the circadian system is deeply involved in renal function.

Adenine-induced tubulointerstitial nephropathy in rodents has been established as a strong model of renal dysfunction without the complications of surgery or increased mortality seen in other CKD models, such as unilateral ureteral obstruction and nephrectomy.[16] Normally, adenine is converted into the uric acid allantoin, which is excreted with urine. However, excess adenine accumulates and is converted into 8-dihydroxy adenine, then eventually 2,8-dihydroxy adenine, and these non-soluble materials crystallize in renal tubules and cause damage. After feeding of an adenine (0.2%)–containing casein-based diet for 2–4 weeks, inflammation and fibrosis are detected in the kidney, and the serum markers of renal dysfunction are elevated (BUN and creatinine).[16] However, the impact of adenine on circadian function is not known and is the focus of the present study. First, we analyzed the impact of CKD on locomotor activity, and video-analyzed sleep behavior, food/water intake, and urine volume. Next, we determined the impact of CKD on the rhythms of clock gene expression measured by PER2 bioluminescence. We also measured rhythms in gene expression using real-time polymerase chain reaction by sampling the kidney every 4 hours through the 24-hour cycle. Finally, we determined if the genetic disruption of the circadian clock (*Clock* mutant) rendered the mice more sensitive to renal damage due to the adenine diet.

RESULTS

Adenine-treated mice exhibit disrupted renal function consistent with CKD

After 2 weeks of the adenine diet (Figure 1a), ICR mice showed decreased body weight compared with control mice (Figure 1b). Indicators of renal function, including BUN, serum and urine creatinine, and the anti-inflammatory hormone corticosterone, were found to be increased in the CKD group (Figure 1c–g). The amount and frequency of water intake was higher in both the day and night (Figure 1h). In contrast, food intake was not changed by adenine treatment (Figure 1i). As with drinking behavior, diminished day–night variations of urine total volume, sodium, and potassium were seen in the adenine-treated mice (Figure 1j). The disruption of the day–night difference in urine volume and its contents suggest that the circadian regulation of renal function is disrupted in the treated mice.

CKD mice exhibit disrupted behavioral activity and sleep rhythms

Next, we measured daily rhythms of locomotor activity and sleep behavior as circadian output using infrared sensors (activity) and video-monitored immobility (sleep), respectively, using C57BL/6N male mice. At first, we confirmed serum and urine creatinine changes in this strain (Supplementary Figure S1A). Compared to control mice, the CKD mice showed less locomotor activity in the night, with an increased number of activity bouts (Figure 2a–c). A 2-way repeated measures analysis of variance run on the waveform (1 hour bins) confirmed significant effects of time ($F[23, 322] = 63.56$, $P < 0.001$) and treatment ($F[1, 14] = 5.11$, $P < 0.001$), and an interaction ($F[23, 322] = 3.32$, $P < 0.001$). The % activity in the light phase was also greater in the CKD group (11.5% ± 1.3% in the control group and 20.8% ± 2.1% in the CKD group, $P < 0.01$ by Student's t test). Despite these changes, the strength of the activity rhythm as measured by periodogram analysis did not vary with treatment (39.0% ± 1.6% in the control group and 32.8% ± 4.0% in the CKD group). A similar reduction of activity level and increase in activity bouts were seen in the male ICR strain (Supplementary Figure S2A–C). The amount of sleep behavior was not impacted by the treatment (Figure 2d and e). The CKD mice had a higher number of sleep bouts in their rest-phase (Figure 2f). Thus, the CKD mice exhibited reduced locomotor activity as well as more fragmented rhythms in sleep.

CKD mice exhibited low-amplitude rhythms in PER2-driven bioluminescence measured from the central circadian clock (SCN)

Rhythms in activity and sleep are controlled by the SCN.[12] Therefore, we next sought to determine whether the PER2 protein rhythms measured in the SCN were disrupted in the CKD mice. Brain slices containing the SCN were prepared from PER2::LUCIFERASE mice (ICR background) treated with the adenine diet. We found that the SCN from the CKD

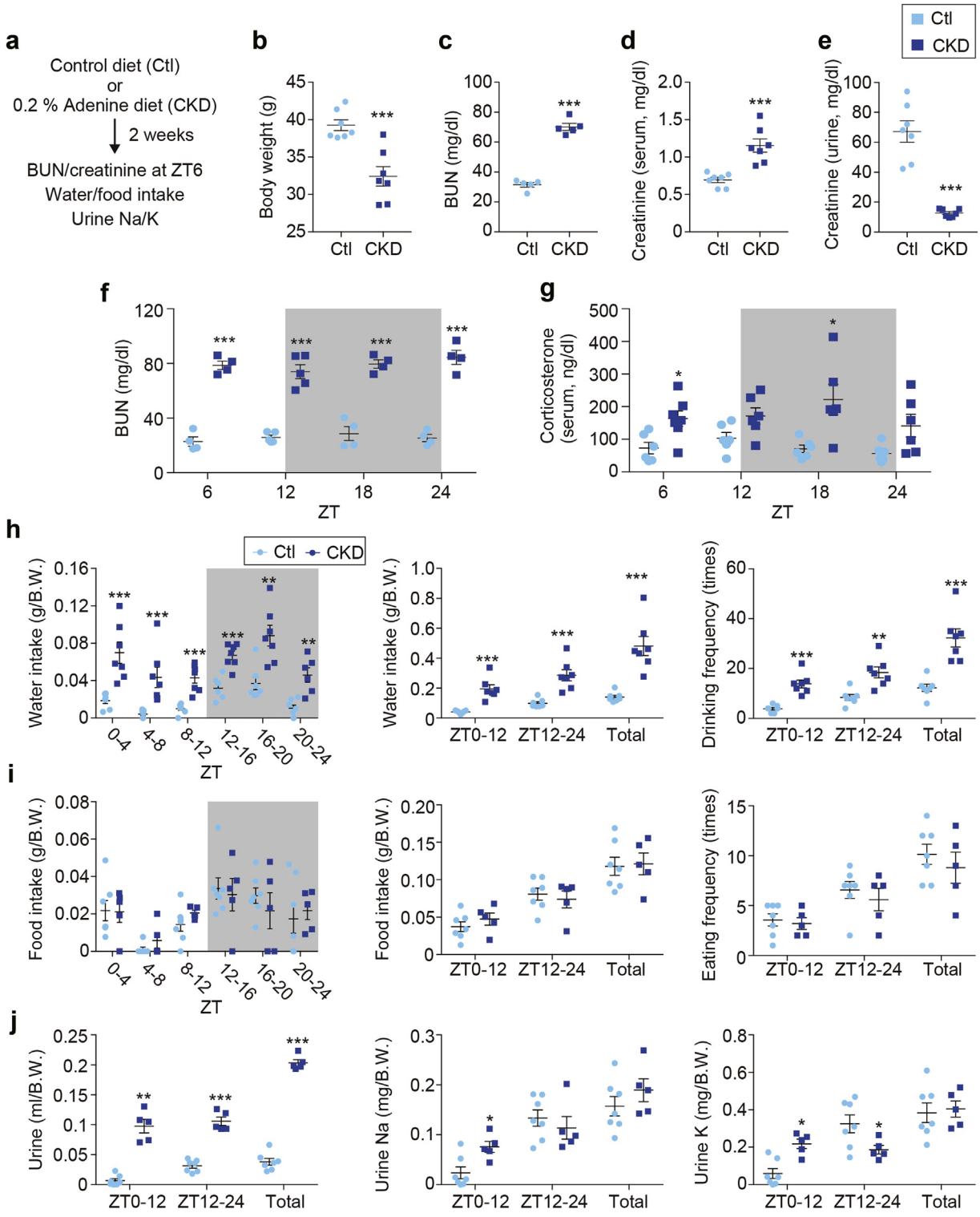

Figure 1 | Day–night differences in water/food intake and urine in adenine-induced mice with adenine-induced chronic kidney disease (CKD). (**a**) Experimental schedule: mice were fed a control (AIN 93N) or adenine (0.2%)-containing diet for 2 weeks. At this point, (**b**) body weight (B.W.), (**c**) blood urea nitrogen (BUN) in serum, (**d**) serum creatinine, and (**e**) urine creatinine were measured at ZT6 (Zeitgeber time; ZT0 is the time of light on and ZT12 is the time of light off); n = 5–7 per group. (**f,g**) BUN and serum corticosterone were measured at 4 time points per day (n = 4 in each for BUN, n = 6 in each for corticosterone). (**h,i**) Water or food intake was measured every 4 hours (left) or 12 hours (middle), and the frequency of intake is shown in the right panel (n = 7 per group). The dark shadows indicate the time of lights-off (ZT12-24). (**j**) Urine volume (left), total urine Na (middle), and total urine K (right) were measured every 12 hours in metabolic cages (n = 5–7 per group). Water, food, and urine are normalized to B.W. All values are expressed as individual plots with mean ± SEM. *P* values, versus control: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

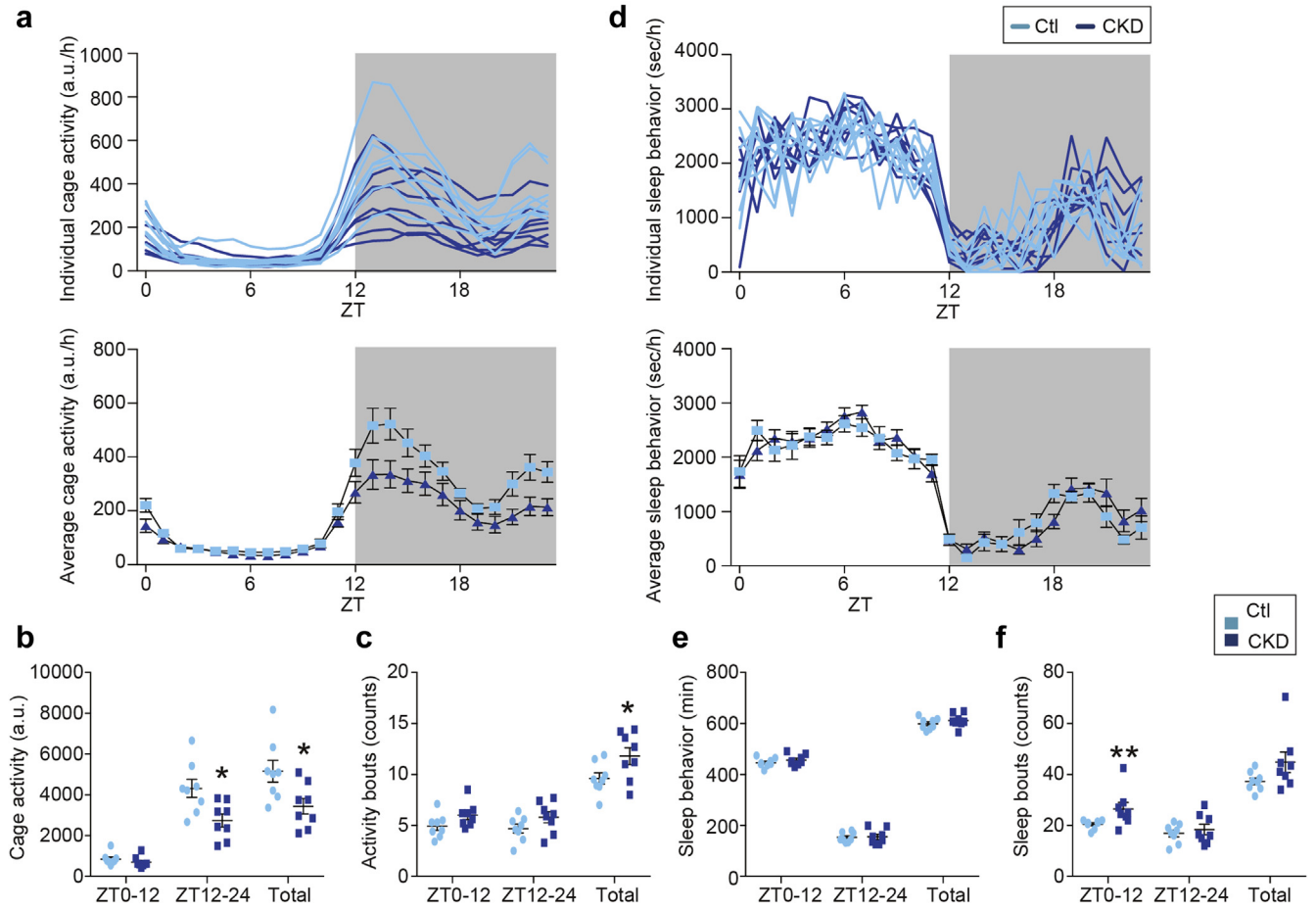

Figure 2 | Cage activity and sleep behavior in adenine-induced mice with adenine-induced chronic kidney disease (CKD). After 2 weeks of control (Ctl) or adenine (CKD) diet treatment to C57BL/6N male mice, cage activity (**a–c**) and sleep behavior (**d–f**) were measured by infrared sensor and video monitoring, respectively (n = 8 in each group). Time-series data of activity or sleep are shown with individual plots (upper panels in **a,d**), 1-hour bins (lower panels in **a,d**), and 12-hour bins (**b,e**). Activity or sleep bout numbers are shown in (**c**) and (**f**). The dark shadows indicate the time of lights-off (Zeitgeber time; ZT0 is the time of light on and ZT12 is the time of light off; ZT12–24). All values are expressed as individual plots with mean ± SEM. *P* values, versus control: **P* < 0.05; ***P* < 0.01.

mice exhibited low-amplitude rhythms that damped faster than that in controls (Figure 3a–e). The endogenous circadian cycle length (period) was not altered, nor did we observe any differences in the peak phase of the bioluminescence rhythms (Figure 3d and e). Our data suggest that CKD weakens the amplitude but not the period or phase of the central circadian clock.

CKD mice exhibited low-amplitude rhythms in PER2-driven bioluminescence measured from peripheral organs *in vivo*

Next, using *in vivo* imaging techniques,[17] we examined PER2::LUC bioluminescence from kidneys, liver, and submandibular gland throughout the 24-hour cycle (Figure 4a and b). We found that the daily average of bioluminescence and amplitude of the rhythms were reduced in all 3 tissues, whereas peak phase was unaltered (Figure 4c–e). We also measured PER2::LUC bioluminescence from kidney explants *in vitro*. Interestingly, we found that the rhythms in bioluminescence were not compromised in the CKD mice under these condition (Supplementary Figure S3). These findings suggest that the molecular clockwork in the intact organism is more impacted by CKD than the clockwork in the isolated kidney. Sex differences of CKD patients and CKD model animals are reported, with greater adverse effects in males.[18,19] Consistently, compared with males (Figures 1 and 4), in female mice (Supplementary Figure S4), we found smaller effects on urine creatinine levels (71% reduction by adenine in females, 81% reduction in males) and *in vivo* renal PER2::LUC rhythms (31% reduction in females, 55% reduction in males).

CKD mice exhibited low-amplitude rhythms in clock gene expression measured from the kidney

To get a more complete view, we used real-time polymerase chain reaction to measure the expression levels of clock genes sampled every 4 hours throughout the 24-hour cycle. The expression levels were reduced at several time points in all clock genes except for *Dec1* and *Cry1* (Figure 5a). In addition, mRNA levels of renal function genes (*Scnn1a, Gilz, Aqp2, V2r*), which were reported to be regulated by the circadian

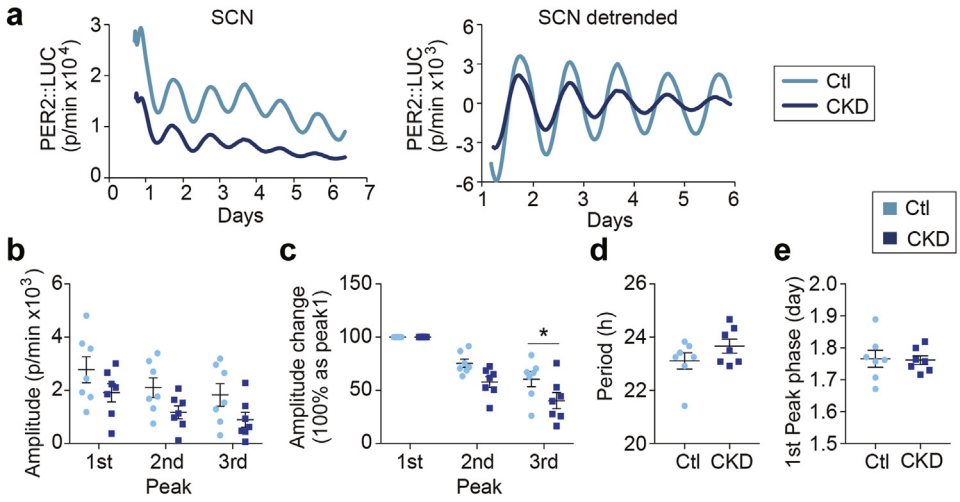

Figure 3 | PER2::LUCIFERASE (PER2::LUC) rhythms in the suprachiasmatic nucleus (SCN) of mice with adenine-induced chronic kidney disease (CKD). After 2 weeks of control (Ctl) or adenine (CKD) diet treatment, PER2::LUC activity was measured *in vitro*. (**a**) Representative PER2::LUC activity (photon [p]/min in a dish) from brain slices containing the SCN. Raw data (left) and detrended data (right) are shown (n = 7 in each group). (**b**) Shown are amplitude, (**c**) amplitude change, (**d**) period, and (**e**) peak phase of the SCN PER2::LUC rhythms. The amplitude of first peak is set as 100% in (**c**). All values are expressed as individual plots with mean ± SEM. *P* values, versus control: $^*P < 0.05$.

clock, also exhibited decreased expression (Figure 5b). Most of the circadian clock or clock-regulated genes that we examined showed significant rhythmicity in both control and CKD conditions (Supplementary Table S1). Some of them showed phase changes by CKD, but the direction of the phase changes depended on the genes (Supplementary Table S1). In contrast, inflammation (*Mmp2, Mmp9, Il-1b*) and fibrosis (*Tgf-b1*) markers were increased significantly throughout the day and night (Figure 5c). Similar results were seen in the C57BL/6N CKD mice for *Rev-erba, Tef, and Tgf-b1*, but not *Per2* (Supplementary Figure S1). The gene expression data indicate that the molecular clock and its rhythmic outputs in the kidney are damped while inflammation is chronically increased in the CKD condition.

Disruption of clock and renal function did not reverse after 2 weeks of recovery

We examined whether the renal gene expression changes in CKD mice would reverse themselves after a return to the normal diet from the adenine diet (Supplementary Figure S5). After 2 weeks of recovery, BUN and urine creatinine levels were slightly recovered but still significantly different from those in control mice. The expression level of clock genes (*Per2, Clock, Rev-erba, Dbp*) and the clock-controlled gene (*Scnn1a*) stayed dampened compared with the controls. *Tgf-b1*, a marker of fibrosis, showed constantly high levels of mRNA as well. These observations suggest that dampened molecular clocks persist after establishment of CKD.

Clock mutation made mice vulnerable to the impact of the adenine diet

A variety of studies suggest that a robust circadian rhythm is important for health and that environmental or genetic disruptions of the circadian system can accelerate disease progression.[12,20,21] Therefore, we examined the impact of $Clock^{\Delta 19/\Delta 19}$ mutation, in which exon 19 (51 amino acids) is missing and dominant-negative protein is expressed,[22] on the development of adenine-induced CKD (Figures 6–8). *Clock* mutant mice showed a significantly lower level of urine creatinine on days 9 and 13, and an earlier increase of serum BUN levels on days 5 and 9 compared with wild-type (WT) mice (Figure 6a and b). Serum creatinine or urine osmolarity were not different between genotypes (Figure 6c and d). Renal hematoxylin and eosin, and Masson trichrome, staining confirmed adenine-induced tubular damage and showed significantly higher 2,8-dehydroxyadenine deposition in *Clock* mutant mice compared to WT mice, but other factors (distal tubular dilation and fibrosis) were not different between genotypes (Figure 6e–g). As reported previously,[13,15] *Clock* mutant mice showed a higher volume of total urine (corrected by body weight) with increased daytime urine excretion in the control diet condition (Figure 6h). The treated mutants exhibited significantly increased urine excretion and urine sodium/potassium content during both day and night, compared with WT CKD mice (Figure 6h). After 2 weeks of adenine treatment, daily gene expression changes were measured (Figure 7a and Supplementary Figure S6A). *Mmp2/9*, but not *Tgf-b1*, expression was significantly increased compared with that in WT mice in CKD conditions. For *Mmp2*, a 2-way analysis of variance revealed significant effects of treatment ($F[1, 60] = 167.2$, $P < 0.001$) and genotype ($F[1, 60] = 10.45$, $P < 0.01$), and an interaction ($F[1, 60] = 4.18$, $P < 0.05$). For *Mmp9*, a 2-way analysis of variance confirmed significant effects of treatment ($F[1, 60] = 106.5$, $P < 0.001$) and genotype ($F[1, 60] = 29.47$, $P < 0.001$), and an interaction ($F[1, 60] = 4.81$, $P < 0.05$). Although the

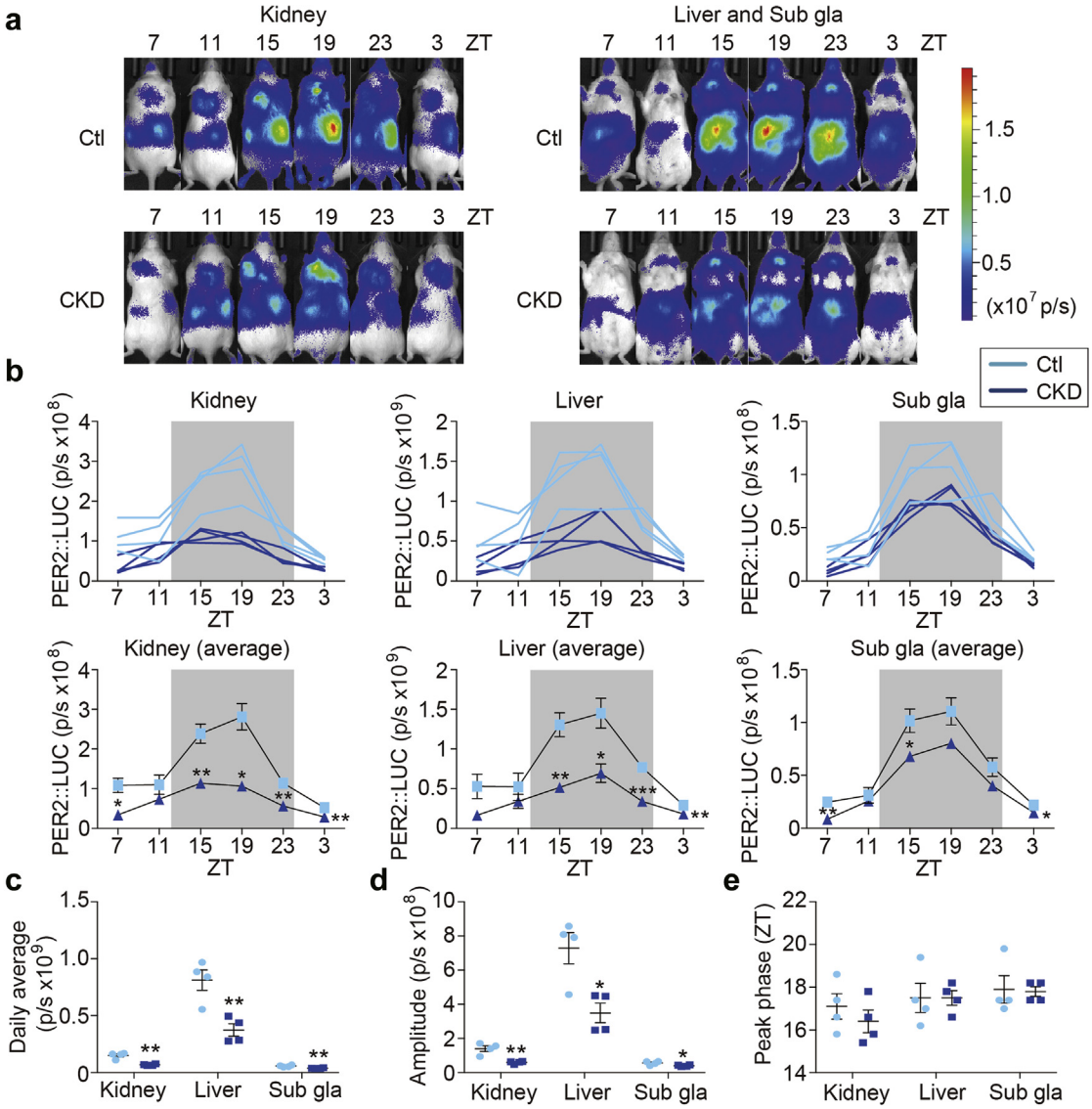

Figure 4 | PER2::LUCIFERASE (PER2::LUC) rhythms in the peripheral tissues of mice with adenine-induced chronic kidney disease (CKD). After 2 weeks of control (Ctl) or adenine (CKD) diet treatment, PER2::LUC activity was measured utilizing *in vivo* monitoring methods. (**a**) Representative photographs of *in vivo* PER2::LUC imaging in the kidney, liver, and submandibular gland (Sub gla) taken every 4 hours are shown (n = 4 in each group). (**b**) Individual (upper) or averaged (lower) PER2::LUC activity rhythms (photon [p]/sec from the tissues) in the peripheral tissues. The dark shadows indicate the time of lights-off (Zeitgeber time; ZT0 is the time of light on and ZT12 is the time of light off; ZT12–24). (**c**) Shown are daily average of bioluminescence, (**d**) amplitude, and (**e**) peak phase of peripheral PER2::LUC rhythms. All values are expressed as individual plots with mean ± SEM. *P* values, versus control: $^{*}P < 0.05$; $^{**}P < 0.01$.

2,8-dehydroxyadenine deposition was higher in the mutant, *Xanthine dehydrogenase* (*Xdh*), which produces insoluble 2,8-dehydroxyadenine, was increased in the liver in both adenine-treated WT and mutant mice with no genotype difference (Figure 7a). Consistently, matrix metalloproteinase 2 (MMP2) protein level and gelatinase activity of pro-MMP2 were higher in the mutant mice, measured by western blotting and zymography, respectively (Figure 7b–d). In summary, our data suggest that the *Clock* mutation potentiated the progression of adenine-induced CKD and led to worsening of symptoms due to the higher 2,8-dehydroxyadenine deposition and MMP2 expression.

Adenine-treated Clock mutant mice exhibited hypertension and reduced heart rate, with disrupted circadian rhythmicity

The effects of adenine treatment and *Clock* mutation on heart rate and blood pressure were examined using a telemetry system and the tail-cuff method (Figure 8). Under control conditions, the *Clock* mutant mice showed a phase delayed rise in diurnal rhythm in blood pressure, heart rate, and activity (Figure 8a; cosinor analysis and 2-way repeated measures analysis of variance are indicated in Supplementary Tables S2 and S3, respectively), as previously reported.[23] After 2 weeks of adenine treatment, systolic blood pressure was increased compared to controls, and the normal

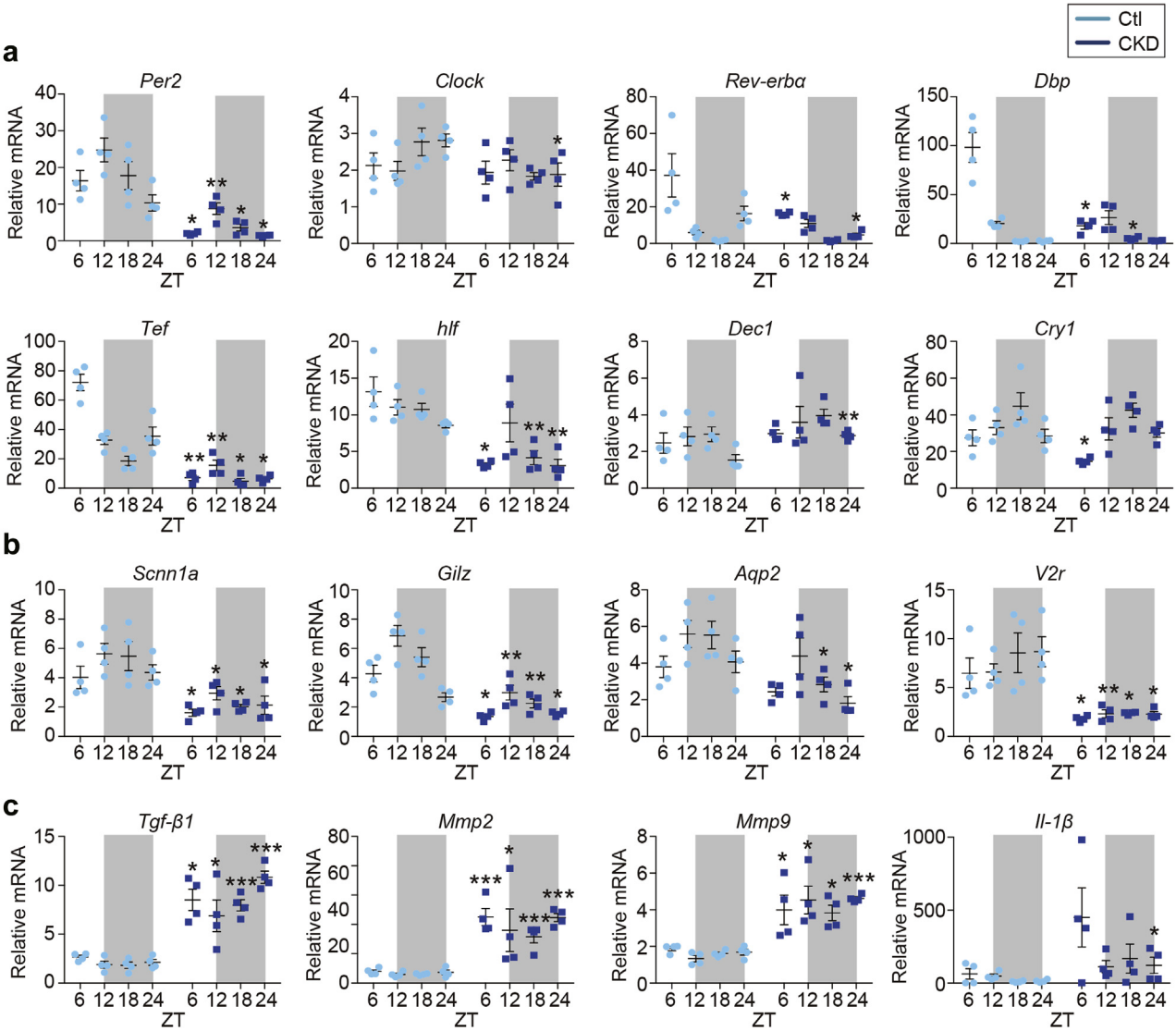

Figure 5 | Renal gene expression rhythms in mice with adenine-induced chronic kidney disease (CKD). Daily mRNA expression of clock genes (**a**), clock-controlled renal genes (**b**), and renal inflammation or fibrosis markers (**c**) were measured after 2 weeks of control (Ctl) or adenine (CKD) diet treatment (n = 4 in each time point). The dark shadows indicate the times of lights-off (Zeitgeber time; ZT0 is the time of light on and ZT12 is the time of light off; ZT12–24). All values are expressed as individual plots with mean ± SEM. *P* values versus control at each time point: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

day–night difference was disrupted with non-dipping at rest phase, analyzed by the goodness-of-fit value of cosinor analysis and the percent difference between day and night (Figure 8a; Supplementary Tables S2 and S3). Hypertension was confirmed using a tail-cuff blood pressure measurement, and the mutant CKD mice showed higher blood pressure compared to WT CKD mice at ZT14-18 (Figure 8b). The heart rate in the mutant mice was lower than that in controls and exhibited a disrupted daily rhythm (Figure 8a). Similar to the locomotor activity data measured by infrared sensor (Figure 2a–c and Supplementary Figure S2), activity levels as measured by telemetry sensor were reduced in CKD condition (Figure 8a). Therefore, *Clock* mutant mice showed higher blood pressure and lower heart rate than WT mice under adenine treatment, confirming that *Clock* mutation makes mice vulnerable to the impact of the adenine diet.

DISCUSSION

This study used the adenine-induced model of kidney damage to determine whether CKD can impact the circadian timing system. We observed that the treated mice exhibited fragmented rhythms in activity and sleep, and reduction of overall activity level. We found significant reductions in the amplitude of the PER2-driven bioluminescence rhythms in the SCN *in vitro* and in the kidney *in vivo*. Further analysis of gene expression in the kidney using real-time polymerase chain reaction confirmed that amplitudes of the oscillations in a number of circadian-regulated transcripts were reduced.

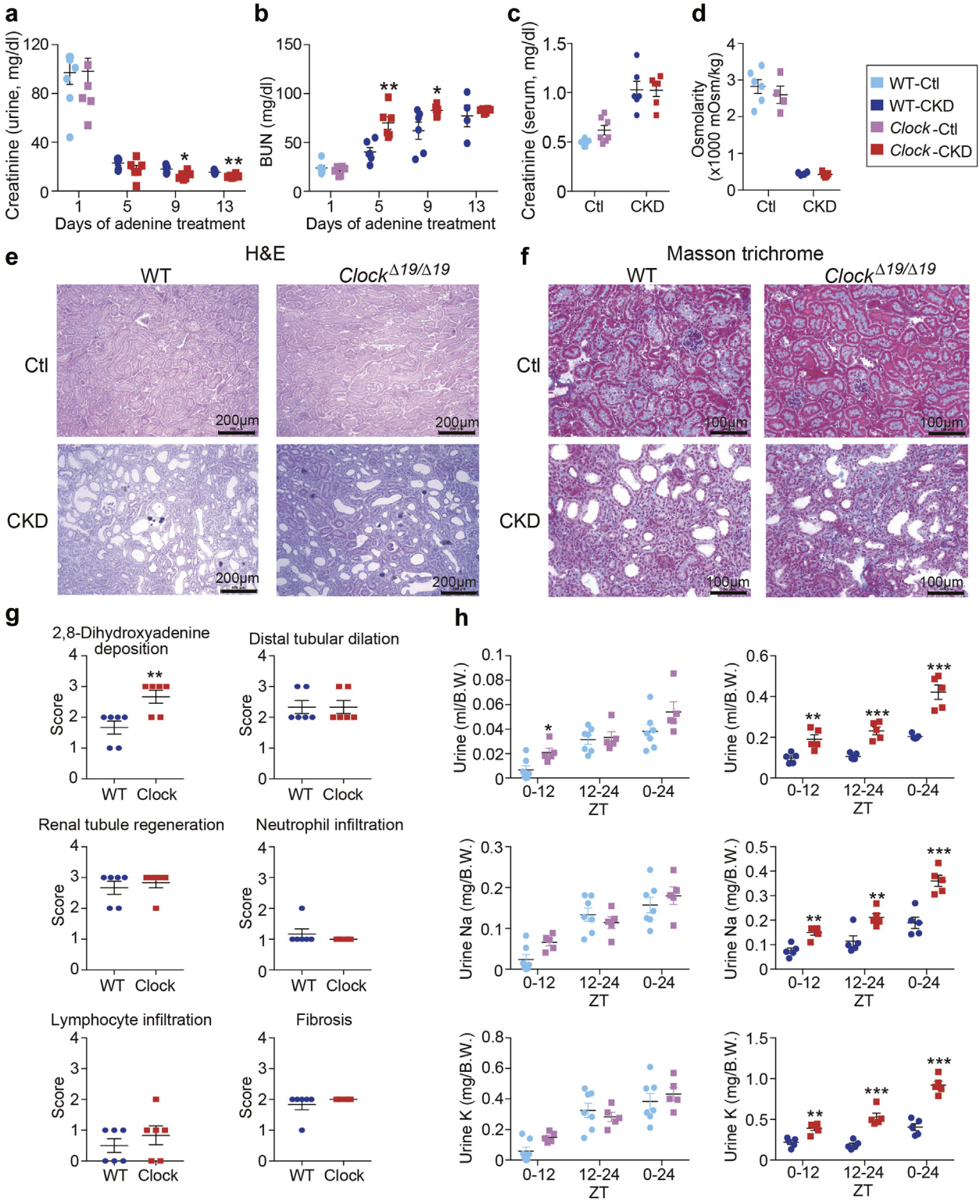

Figure 6 | Urine and kidney histology of adenine-induced chronic kidney disease (CKD) in Clock mutant mice. (**a,b**) During the 2 weeks of adenine (0.2%, CKD) treatment, urine creatinine and blood urea nitrogen (BUN) were measured every 4 days from wild-type (WT) and Clock mutant mice (n = 6 in each, except WT on day 13 for n = 4). (**c,d**) After 2 weeks of adenine treatment, serum creatinine and urine osmolarity were measured at ZT6 (Zeitgeber time; ZT0 is the time of light on and ZT12 is the time of light off). (**e,f**) Representative kidney images of hematoxylin and eosin (H&E; **e**) and Masson trichrome staining (**f**) in each condition. (**g**) Averaged score of each pathology finding (score level 1 for normal and level 5 for worst) in CKD mice. (**h**) After 2 weeks of control (Ctl) or adenine treatment, total urine volume and urine Na/K were measured in the light and dark phase by using metabolic cages (n = 7 for Ctl, n = 5 for CKD in each genotype). All values are expressed as individual plots with mean ± SEM. *P* values, versus WT: **P* < 0.05; ***P* < 0.01; ****P* < 0.001. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

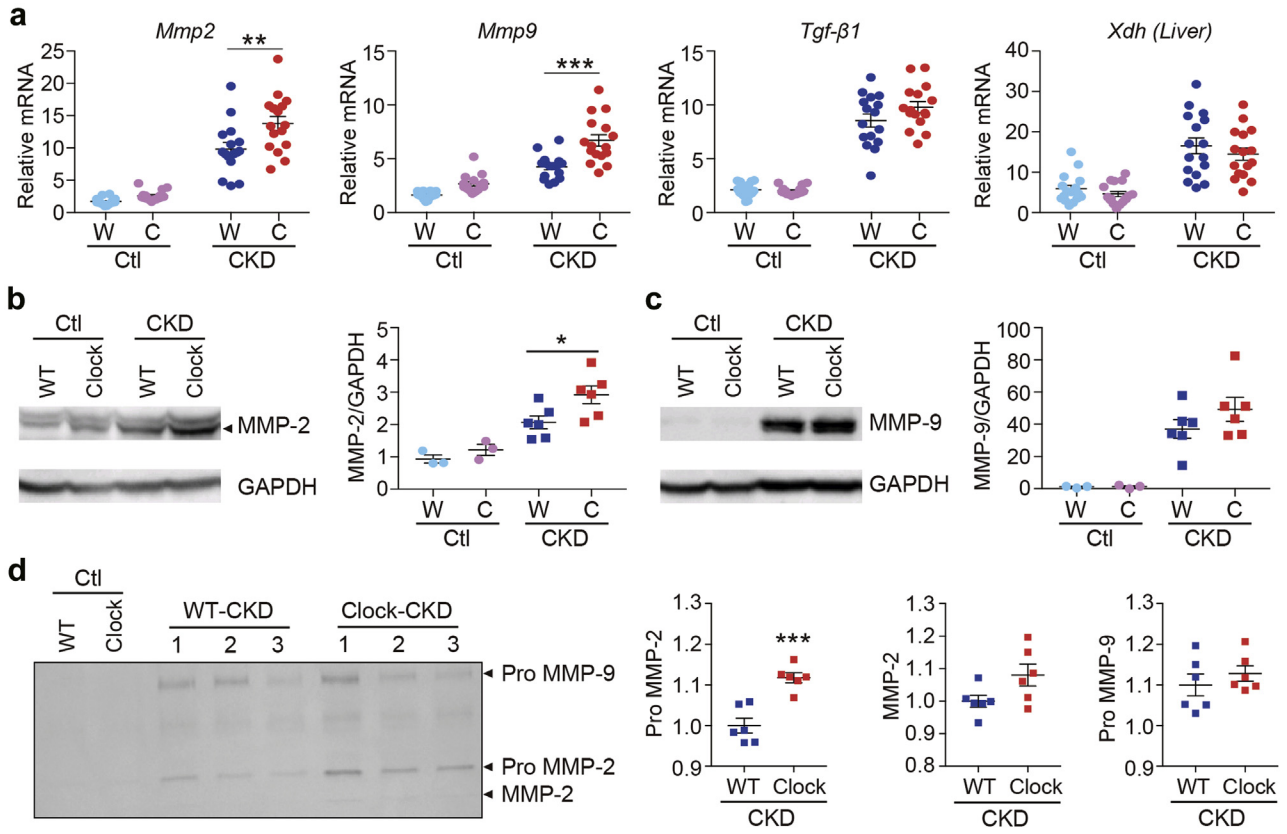

Figure 7 | Matrix metalloproteinase (MMP)–2 and –9 expressions of adenine-induced chronic kidney disease (CKD) in Clock (C) mutant mice. (**a**) mRNA gene expressions (*Mmp-2*, *Mmp-9*, and *Tgf-β1* in the kidney; *Xdh* in the liver) after 2 weeks of adenine treatment in wild-type (WT; W) and Clock mutant mice. Samples were taken at ZT6, Z12, Z18, and Z24 (Zeitgeber time; ZT0 is the time of light on and ZT12 is the time of light off; n = 4 in each time point) but the all–time point samples were combined. Daily change of these gene expressions are shown in Supplementary Figure S6. (**b,c**) Western blotting analysis of MMP-2 (**b**) and MMP-9 (**c**) in the kidney are shown with representative band images (left) and relative values (right, 1 as WT mice with control [Ctl] diet). (**d**) Gelatin zymography of MMP-2 and MMP-9 in the kidney with representative band images (left) and relative values (right, 1 as WT mice with Ctl diet). n = 3 in Ctl, n = 6 in CKD for western blotting and zymography. All values are expressed as individual plots with mean ± SEM. *P* values, versus WT: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

The CKD model also exhibited clear evidence of renal inflammation and distal tubular dilation. Non-dipping hypertension with disrupted circadian rhythm and low heart rate was seen in the CKD model mice, confirming the circadian disruption in this model and similarity with human CKD patients.[24] Finally, *Clock* mutant mice with a dysfunctional circadian timing system proved to be more sensitive to adenine-induced kidney damage than were WT controls, which is caused by higher adenine metabolite deposition and higher MMP expressions in the kidney.

Previous papers have shown that renal disease patients have sleep problems, including fragmentation of sleep[6] and daytime sleepiness[7] with reduced duration and intensity of daytime physical activity.[6] We found that the adenine-treated mice did not exhibit extensive disruption or increase of their rhythm in sleep. We did see clear evidence of fragmentation of the rhythms in sleep and activity with decreased cage activity (Figure 2; Supplementary Figure S2). Prior work in another CKD model (5/6 nephrectomy rats) found decreased cage activity and increased rapid eye movement/non–rapid eye movement (REM/NREM) sleep in the end of the active period, but it did not examine the fragmentation of sleep and activity.[25] Fragmented sleep in the rest period could be due to the arousal signals of urine excretion or thirst in the CKD mice, as the treated mice did exhibit more urination and water-drinking behavior during their rest period. These effects are due to the downregulation of *Aqp2* and *V2r* gene expression in both a day– and night–24 hour period in the adenine-treated kidney (Figure 5b). These results are consistent with a previous report in which acute adenine intake (7 days) directly downregulates *Aqp2*, *V2r*, and *Nkcc2* gene expression and causes diabetes insipidus.[26]

We found that the treated mice exhibited reduced amplitude without change of the cycle length (period) or phase in the rhythm of PER2::LUC activity in the SCN, *in vitro* (Figure 3). There may be a strain difference in the clock gene expressions in the SCN of CKD mice. Recently, Myung *et al.*[27] reported no effect on the PER2::LUC rhythms of adenine-

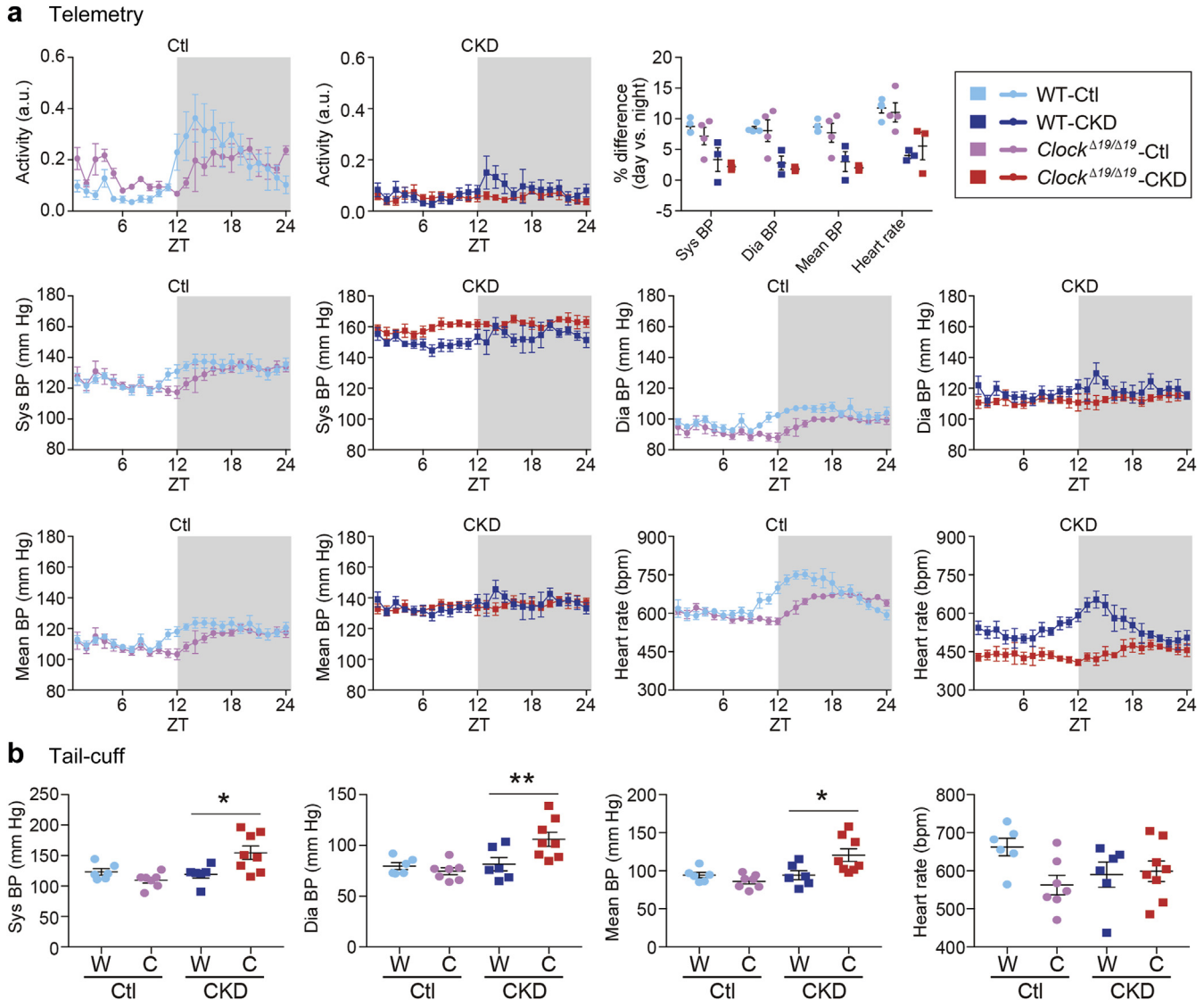

Figure 8 | Blood pressure (BP) measurement of adenine-induced chronic kidney disease (CKD) in Clock (C) mutant mice. (**a**) Averaged systolic (Sys), mean, and diastolic (Dia) BP, heart rate (beats per minute [bpm]), and movement (activity) measured by the radiotelemetry method after 2 weeks of control (Ctl) or adenine (CKD) diet treatment are shown. n = 4 for Ctl, n = 3 for CKD in each genotype. Percent difference of each parameter between day and night is also shown. Individual plots are shown in Supplementary Figure S6B. Results of cosinor analysis and 2-way repeated measures analysis of variance are shown in Supplementary Tables S2 and S3, respectively. (**b**) Averaged Sys, mean, and Dia BP, and heart rate measured by tail-cuff method at ZT14–18 (Zeitgeber time; ZT0 is the time of light on and ZT12 is the time of light off; data measured at ZT4–8 are shown in Supplementary Figure S6C). All values are expressed as individual plots with mean ± SEM. *P* values, versus wild-type (WT; W): $^{*}P < 0.05$; $^{**}P < 0.01$.

treated C57BL/6J male mice, although these mice showed similar disruption in locomotor activity. We do not know the mechanisms by which peripheral kidney damage can impact the SCN. Increased depression-like behavior has been reported in adenine-induced CKD mice and 5/6 nephrectomy mice.[28,29] Interestingly, major depression has been found to be associated with reduced gene expression rhythms in the human cortex.[30] Furthermore, the adenine treatment causes systemic inflammation in mice and rats.[19,31] A variety of studies[25] have shown that chronic inflammation can impact the SCN and specifically reduce the amplitude of rhythms in clock gene expression in this structure.[32,33]

The present study uncovered strong evidence that adenine-induced CKD model mice exhibited disrupted circadian rhythms in the kidney, liver, and submandibular gland, using *in vivo* imaging (Figure 4). Using real-time polymerase chain reaction, we demonstrated that the low-amplitude rhythms were seen in a variety of genes, suggesting that the circadian clockwork is compromised in the kidney of our CKD model in ICR and C57BL/6N mice (Figure 5; Supplementary Figure S1). Although we did not see the same reduction *in vitro* PER2::LUC rhythms (Supplementary Figure S3), this is because the clock resetting effect might have happened when the explants were put into the fresh recording medium, and resetting can happen

more frequently if the oscillation is weakened *in vivo*.[17] However, this result suggests that adenine-treated kidney can oscillate normally *in vitro* in the fresh medium but not *in vivo*. Prior work examining clock gene expression using other CKD models (5/6 nephrectomy mice or rats) found less-consistent effects. In those models, the amplitudes of the daily expression levels in renal *Per1* and *Per2* mRNA were increased in the mouse model and were unchanged in the rat model, and the renal *Clock* mRNA level was increased throughout the entire day in the ICR mouse model.[34,35] As with the 5/6 nephrectomy model, an increased amplitude of renal *Per2* expression rhythm was reported in unilateral ureteral obstruction–induced CKD C57BL/6J mice.[36] In contrast to the 5/6 nephrectomy or ureteral obstruction models, but consistent with our findings, cisplatin-induced acute kidney injury in the proximal straight and distal convoluted tubules showed significant reduction of renal *Per2* mRNA oscillation 72–92 hours after cisplatin injection in rats and C57BL/6J mice.[37,38] Although it is difficult to explain according to strain or species, this discrepancy among CKD models might result from the pathology differences among the models. Although all CKD models develop decreased creatinine and BUN clearance with renal fibrosis, 5/6 nephrectomy induces tubular atrophy, tubulointerstitial fibrosis, and glomerulosclerosis, whereas adenine treatment mainly induces tubulointerstitial damage with infiltrating leukocytes, interstitial edema, and widening of Bowman's space.[16,19] In addition, renal weight is increased after both 5/6 nephrectomy and ureteral obstruction, but it is decreased by adenine treatment. Taken together, reduction of *Per2* mRNA in the adenine- or cisplatin-treated kidney might be a reflection of the damage of convoluted tubules in the medulla of the kidney, but more work is required to investigate this possibility.

The current study is the first to demonstrate accelerated adenine-induced CKD development in *Clock* mutant mice with increased MMPs and 2,8-dehydroxyadenine deposition in the kidney, disrupted water/Na/K holding, higher blood pressure, and lower heart rate, compared with WT mice. Renal fibrosis was more severe in CLOCK-deficient mice with unilateral ureteral obstruction–induced CKD, compared with WT CKD mice.[36] In this model, extracellular matrix–related mRNA of *Col1A1*, *Col4A1*, and *Ctgf*, and *Tgf-b1*, were significantly higher in the kidney of CLOCK-deficient CKD mice. Increased expression of COX1/2 by transforming growth factor-β–enhanced oxidative stress in CLOCK-deficient mice resulted in accelerated CKD development.[36] MMP2 and 9 matrix metalloproteinases regulate the extracellular matrix and are related to many signals, including inflammation, oxidative stress, growth factor, and monocyte chemoattractant proteins, and they are elevated in CKD patients.[39] In *Bmal1*-KO mice, MMP2 and 9 were upregulated in remodeled arteries after arterial ligation.[40] Cardiomyocyte-specific *Bmal1* knockout also increased MMP9 in the heart, with accelerated aging and increased mortality.[41] Thus, dysregulated MMP expression in circadian clock disruption led to CKD development in the current study. Consistent with these biochemical changes, blood pressure was increased whereas heart rate was reduced in adenine-treated *Clock* mutant mice. Cardiovascular events are prevalent and increase mortality in CKD patients[42] and in shift workers.[43] Therefore, our data, along with the established literature, support the hypothesis that the circadian disruption resulting from CKD may further exacerbate progression of the disease.

In conclusion, the present study examined the reciprocal relationship between circadian clocks and adenine-induced renal dysfunction. Because young (age 2–3 months) male mice were used in this study, the impact of aging and sex differences are important topics for future work. Another limitation of this study is that we used a 2-week treatment that was not sufficient to induce fibrosis in the kidney. Our findings do help us understand sleep and rhythm disturbances in adenine phosphoribosyltransferase deficiency, which is a rare inherited metabolic disorder that leads to the accumulation of 2,8- dihydroxyadenine and causes kidney failure.[44] More broadly, the results suggest that circadian disruption caused by environmental factors, such as nighttime shift work or jet lag, may be a risk factor for CKD development. In fact, female shift workers are reported to have an increased risk of CKD.[45] Finally, we speculate that treating the circadian dysfunction in the kidney could be helpful in ameliorating additional disease progression in CKD. Many studies have shown that lifestyle changes, such as light exposure, eating time, and exercise, enhance the circadian system[46] and could be recommended to improve kidney health.

METHODS

Animals

All animal care and procedures, except cage activity and sleep analysis, were in accordance with the guidelines of the Committee for Animal Experimentation of the School of Science and Engineering at Waseda University and in compliance with the law (No. 105) passed by and notification (No. 6) of the Japanese government. Experiments were approved by the School of Science and Engineering at Waseda University. The cage activity and sleep analysis shown in Figure 2 were conducted at the University of California—Los Angeles (UCLA) using C57BL/6N male mice purchased from the UCLA animal core facility. The experimental protocols used in this study were approved by the UCLA Animal Research Committee. The UCLA Division of Laboratory Animals recommendations for animal use and welfare, as well as the National Institutes of Health guidelines, were followed. ICR mice, homozygous *Clock*$^{\Delta 19/\Delta 19}$ mutant mice[22] on the ICR background (originally C57BL/6J background, but crossed over 15 times with ICR mice at Waseda University from 2004), and heterozygous PER2::LUC knock-in mice[47] on the ICR background were used in this study, except for the above-mentioned sleep analysis. Mice were maintained on a 12:12 light/dark cycle (with lights on at 08:00) at room temperature (23 °C ± 1 °C), 60% ± 5% humidity. Male mice (age 2–4 months) were used in this study, except for those in Supplementary Figure S4.

CKD model mice

We used a previously described model of adenine-induced tubulointerstitial nephropathy in mice.[16] In brief, CKD group mice were fed 0.2% adenine mixed with AIN-93M (Research Diets, Inc., New

Brunswick, NJ) for 2 weeks. Control-group mice were fed the normal AIN-93M diet. Powder diets were placed on the bottom of the cage in the food container in most experiments, except that pellet-type diets were used for measuring feeding pattern to improve accuracy. More detailed information is in the Supplementary Methods.

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

HM, YT, DW, HB-W, TY, HW, AH, MY, HM, KH, HS, TS, RH, KT, MT, SM, and SA performed the experiments. HM, YT, CSC, and SS designed the experiments, analyzed the data, and wrote the article. DW and HW helped write the article.

SUPPLEMENTARY MATERIAL

Supplementary Methods.

Figure S1. Serum/urine creatinine and renal gene expression changes by adenine-induced CKD in C57BL/6N male mice.

Figure S2. Cage activity in mice with adenine-induced CKD.

Figure S3. PER2::LUCIFERASE rhythms in the kidney of mice with adenine-induced CKD.

Figure S4. PER2::LUCIFERASE rhythms in the peripheral tissues of female mice with adenine-induced CKD.

Figure S5. Renal gene expression rhythms after discontinuing the adenine diet.

Figure S6. Adenine-induced CKD in Clock mutant mice.

Table S1. Achrophase and goodness-of-fit value evaluated by cosinor analysis.

Table S2. Cosinor analysis of the telemetry data.

Table S3. Results of 2-way repeated measures ANOVA of the telemetry data.

Table S4. Primer list.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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