ARTICLE

The adrenal gland circadian clock exhibits a distinct phase advance in spontaneously hypertensive rats

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Abstract

The circadian clock influences a multitude of cellular and biological processes, including blood pressure control. Spontaneously hypertensive rats (SHR) exhibit aberrant circadian rhythms affecting cardiovascular parameters, and they also have abnormal clock gene expression profiles in several organs. Given the important role of the adrenal gland in orchestrating circadian oscillations, we investigated the adrenal gland circadian clock in SHR and control Wistar-Kyoto rats maintained under a 12-hour light–dark cycle. Adrenal glands, livers, and serum samples were collected every 4 h and mRNA was extracted for analysis of clock gene expression. Serum levels of corticosterone and aldosterone were also analyzed. Overall, the circadian profiles of *Bmal1*, *Per2*, *Per3*, *Cry1*, *RevErba*, *Revervb,* and *Dbp* gene expression in SHR adrenal glands were phase-advanced relative to controls. The expression profile of *StAR* (a representative gene under circadian control in the adrenal gland), as well as the circadian rhythms of serum concentrations of corticosteroid and aldosterone were also phase advanced. *E4bp4* gene expression was significantly higher during the dark period, yet the expression of its transcriptional activator, *Rora*, was significantly lower throughout the 24 h period in SHR adrenal glands than in controls. This paradoxical high *E4bp4* gene expression was, however, not observed in the liver. In addition, *Per1*, *Per2*, *Per3*, *Reverba*, and *Reverbb* mRNA tended to be lower in SHR adrenal glands than in controls. Thus, we conclude that SHR possess an abnormal adrenal circadian clock, which may affect the transcriptional regulation of clock-controlled genes, and steroid hormone secretion by the adrenal gland.

Key words Adrenal gland · Circadian clock · Hypertension · SHR · Rats · Spontaneous hypertensive

Introduction

A large number of molecular, cellular, and behavioral processes exhibit circadian (daily) oscillation that is entrained by external Zeitgebers. In mammals, the endogenous oscillator receiving Zeitgeber information resides in the suprachiasmatic nucleus (SCN) of the hypothalamus [1]. One complete circadian oscillation has a period of ~ 24 h, and evolved to permit optimal behavior in response to the daily light cycle [1]. Circadian neural and humoral signals generated by the SCN coordinate rhythmicity in other, peripheral tissues, most

of which exhibit robust circadian rhythms at the gene expression and cellular function level. They are considered to be "peripheral clocks", providing diurnal biological output that is essentially coordinated by SCN-mediated signaling, and thus the external environment [1].

The molecular mechanisms underpinning the circadian clock are transcription–translation feedback loops that together generate a 24 h oscillation in gene and protein expression [2]. In brief, the core feedback loop is centered on the basic helix-loop-helix-PAS transcription factors, circadian locomotor output cycles kaput (CLOCK) and brain and muscle Arnt-like protein-1 (BMAL1). CLOCK and BMAL1 heterodimerize and bind E-box elements found in the promoters of their target genes, including *Period* (*Per1*, *Per2,* and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*), to activate their transcription [1, 2]. The PER and CRY proteins also form a complex together, which ultimately inhibits CLOCK–BMAL1 activity and thus constitutes a negative feedback loop [1, 2]. *Chrono*, a recently

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discovered clock gene found to interact with the central circadian oscillator, independently inhibits CLOCK–BMAL1 activity [3].

The central circadian feedback loop is modulated and fine-tuned by accessory feedback loops based on the rev response element (RRE) and D-box element. *Rora* and *Reverb* genes contain an E-box and encode a transcriptional RRE activator and repressor, respectively. In this manner, *Rora* and *Reverb* provide an extra layer of regulation to *Bmal1*, *Clock*, *Cry*, and *E4bp4* gene expression [2]. *Dbp* is a D-box-positive regulator and its transcription is also regulated by an E-box element in its promoter. Conversely, *E4bp4* is a D-box-negative regulator, and its gene transcription is regulated by an RRE. Together, these genes modulate the transcription of genes carrying D-box elements [2].

The overall output of the molecular circadian feedback loop is daily, biphasic transcriptional rhythms of a large number of "clock-controlled genes" (CCGs) carrying E-box, D-box elements or RRE [1, 2, 4]. For instance, the daily cycle of glucocorticoid secretion from the adrenal gland depends on the E-box-mediated transcription of *StAR*, which encodes the rate-limiting enzyme for glucocorticoid synthesis [1, 5]. Rhythmic glucocorticoid secretion is one humoral factor involved in maintaining the circadian clock; thus the peripheral adrenal gland clock likely has an important role in coordinating other peripheral clocks in the body [1, 5].

The circadian clock has various links with the cardiovascular system in mammals, including in humans [6–9]. Improving our understanding of how the circadian clock interacts with blood pressure regulatory mechanisms has important clinical relevance. This is because perturbed daily rhythms in blood pressure can be independent risk factors for end-organ damage [10–12].

Spontaneously hypertensive rat (SHR) is a valuable model of essential hypertension. Relative to normotensive Wister Kyoto (WKY) rats, SHR exhibit perturbations in their rhythmic cardiovascular parameters, such as blood pressure and heart rate [13–16]. Abnormal circadian oscillations in the SCN and several peripheral tissues have been reported in SHR, but to date, circadian analysis of the adrenal gland in this rat model has not been reported [14, 15]. The present study aimed to investigate the circadian profile of the adrenal gland of SHR at the genetic level.

Methods

Ethics approval

Our investigation conformed to the Guide for the Care and Use of Laboratory Animals, 8th Edition. All research protocols in this study involving animals were examined and approved by the Ethics Committee for Animal Experiments of Nihon University School of Medicine (AP14M049).

Experimental animals

Male SHR/Izm and WKY/Izm rats (aged 4-weeks) were purchased from the Disease Model Cooperative Research Association (Kyoto, Japan). All rats were maintained under a 12-hour light–dark cycle (lights on at 0800 h or Zeitgeber time (ZT) 0, and lights off at 2000 h or ZT12) at a light intensity of 160 ± 10 lux, a constant temperature of $22 \pm 2\,^{\circ}$C and a humidity of $55 \pm 5\%$ until 5-week old before use in subsequent experiments. A standard chow diet and water was available ad libitum.

Blood pressure measurement

Blood pressure, heart rates, and body weights of SHR/Izm and WKY/Izm rats ($n = 3$ per group) were measured at ZT0 to ZT4. The tail cuff method using Softron BP-98A (Softron Co., Tokyo, Japan) was employed for blood pressure and heart rate measurements. Values represent mean ± standard error of the mean. Statistically significant differences between groups were calculated by Welch's t test, with $P < 0.05$ considered significant.

Tissue sampling

Rats were anesthetized with 4–5% isoflurane and then maintained with 2–3% isoflurane inhalation. SHR/Izm and WKY/Izm animals were killed every 4 h (ZT0, 4, 8, 12, 16, and 20) and their adrenal glands, livers, and inferior vena cava blood samples were collected. The adrenal glands and the livers were immediately frozen and kept at $-80\,^{\circ}$C until use. Serum fractions were isolated from whole blood samples, and also frozen and maintained at $-80\,^{\circ}$C until use.

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR) assays

RT-qPCR was performed to evaluate circadian clock gene and *StAR* expression by SHR/Izm and WKY/Izm animals ($n = 5$ per group). Adrenal glands were homogenized using a TaKaRa BioMasher Standard (Takara Bio Inc., Otsu, Shiga, Japan), and liver samples were powdered using a Cryo-Press (Microtec Co., Ltd., Funabashi, Chiba, Japan) before total RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The RNA samples were treated with deoxyribonuclease I, Amplification Grade (Invitrogen), and cDNA was generated using a High Capacity cDNA Reverse Transcription Kit (Applied

Biosystems, Foster City, CA, USA). cDNA was amplified in triplicate on a Step One Plus Real-Time PCR system (Applied Biosystems).

For Taqman PCR assays, amplifications were performed in a 15 μL reaction mix containing 7.5 μL Premix Ex Taq, ROX plus (Takara Bio Inc.), 50 ng cDNA, and 900 nM primer. Primer details are shown in Table 1. The reaction mix was incubated for 20 s at 95 °C, followed by 50 cycles consisting of 1 s at 95 °C and 20 s at 60 °C.

For SYBR green PCR assays, amplification was performed in a 15 μL reaction mix containing 7.5 μL Universal SYBR Select Master Mix (Applied Biosystems), 50 ng cDNA, 400 nM forward and reverse primer set. The primer sequences are shown in Table 1. The reaction mix was incubated for 2 min at 50 °C, 10 min at 95 °C, and then subjected to 50 PCR cycles consisting of 15 s at 95 °C and 1 min at 60 °C.

Gene expression values were quantified by the standard curve method. Target gene expression was normalized against *Gapdh* as the internal control.

Table 1 Primer list

Bmal1	Rn00577590_m1	
Chrono	AJ39RU8	
Clock	Rn00573120_m1	
Cry1	Rn01503064_m1	
Cry2	Rn01485701_m1	
Dbp	Rn00497539_m1	
E4bp4	Rn01434874_s1	
Per1	Rn01496757_m1	
Per2	Rn01427704_m1	
Per3	Rn00709499_m1	
Reverba	Rn01460662_m1	
Reverbb	Rn00596011_m1	
Ror	Rn01173769_m1	
Star	Rn00580695_m1	
Gapdh	Forward	5′- CAACTCCCTCAAGATTGTCAGCAA -3′
	Reverse	5′- GGCATGGACTGTGGTCATGA -3′

For Taqman assay, assay ID of primers purchased from Applied Biosystems (USA) are shown. For SYBR green assay of *Gapdh* gene, forward and reverse primer sequences are shown

Data represent the mean ± SEM. Differences between groups were calculated by Mann–Whitney U test, with a $P < 0.05$ considered statistically significant.

Evaluation of diurnal steroid levels

Serum aldosterone and corticosterone levels of SHR/Izm and WKY/Izm rats ($n = 3$ per group) at ZT0, 4, 8, 12, 16, and 20 were quantified. Serum aldosterone levels were measured by the immunoradiometric method, using SPAC-S Aldosterone Kits (Fuji Inc., Tokyo, Japan). Serum corticosterone levels were measured by enzyme-linked immunosorbent assays using Corticosterone ELISA Kits (Assaypro, St Charles, MO, USA). Values represent mean ± SEM. Differences between groups were calculated by Welch's t test and $P < 0.05$ was taken as statistically significant.

Results

Cardiovascular parameters and body weights of SHR and WKY rats

Blood pressure, heart rates, and body weights of SHR and WKY animals are shown in Table 2. SHR had higher systolic blood pressure, and tended to have higher heart rates and lower body weights than WKY rats. However, only systolic blood pressure differences were statistically significant.

Core clock gene and CCG expression is phase advanced in the SHR adrenal gland

We first analyzed the mRNA expression pattern of the clock genes *Bmal1* and *Clock* (representing E-box transcriptional activators) and *StAR* (a representative E-box-mediated CCG) in the adrenal gland of SHR and WKY rats (Fig. 1). A clear circadian oscillation in gene expression was observed in *Bmal1* and *StAR*. Interestingly, the peak and trough of *Bmal1* expression was advanced by ~ 4 h in SHR relative to WKY rats. The circadian peak of *StAR* was also advanced, but the timing of mRNA decline occurred at the beginning of the dark period in both SHR and WKY rats.

Table 2 Cardiovascular parameters and body weights

	SHR/Izm ($n = 3$)	WKY/Izm ($n = 3$)	P value
Body weight (g)	112.3 ± 0.3	118.0 ± 1.5	0.06
Systolic blood pressure (mm Hg)	145.0 ± 3.6	122.0 ± 5.1	0.026
Diastolic blood pressure (mm Hg)	76.0 ± 5.0	80.7 ± 0.9	0.45
Heart rates (bpm)	454.0 ± 10.7	415.3 ± 14.3	0.10

Values represent mean ± standard error of the mean
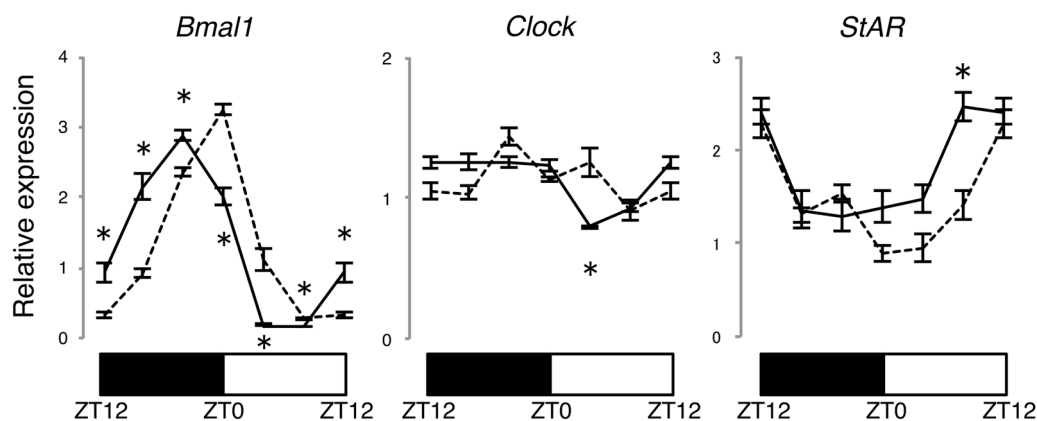
Fig. 1 Diurnal gene expression analysis of *Bmal1*, *Clock*, and *StAR* in the adrenal gland. Diurnal expression patterns of *Bmal1*, *Clock*, and *StAR* in the adrenal gland of SHR (solid line) and WKY (dashed line) rats. The horizontal bar indicates the time course: black indicates the 12 h dark period from ZT12 (2000 h) to ZT0 (0800 h); white indicates the 12 h light period from ZT0 (0800 h) to ZT12 (2000 h). The vertical axis indicates the relative gene expression levels normalized to *Gapdh*. The data represent the means ± standard error of the mean. ZT, Zeitgeber time. *$P < 0.05$

Clock does not typically exhibit a strong circadian rhythm but instead has a relatively constant mRNA expression level across a 24 h period. However, the nadir in *Clock* mRNA expression seemed to occur ~ 4 h earlier in SHR than WKY rats.

We next assessed the mRNA expression pattern of the core clock transcriptional suppressors *Per1-3, Cry1-2*, and *Chrono* (Fig. 2). WKY rats and SHR exhibited clear circadian oscillations in the expression of all six analyzed genes, except for *Per1* in SHR, which had a suppressed amplitude. *Per2* and *Cry1* gene expression was phase advanced by ~ 4 h in SHR, and an advanced decline of *Per3* gene expression was detected in SHR relative to WKY rats. In addition, the overall peak expression level of *Per1*, *Per2*, and *Per3* tended to be lower in SHR. Conversely, *Cry2* gene expression at ZT20 was significantly higher in SHR than in WKY animals. Overall, however, the differences in circadian phase and expression level were minor between SHR and WKY rats, and only a minimal difference was observed in *Chrono* gene expression between the two groups.

Reverba and Reverbb gene expression is phase advanced in SHR adrenal glands

Our next analysis focused on the circadian gene expression of *Rora, Reverba,* and *Reverbb*, which modulate transcription via the RRE (Fig. 3). *Rora* showed a clear circadian oscillation in WKY rats, but gene expression was significantly suppressed in SHR across the 24 h period, except for ZT8. Clear circadian oscillations were detected in *Reverba* and *Reverbb* in both SHR and controls; however, *Reverba* and *Reverbb* gene expression showed a phase advance of ~ 4 h in SHR. Overall, *Reverba* gene expression tended to be lower in SHR than WKY rats.

## Dbp and E4bp4 show an advanced phase in the SHR adrenal gland

Finally, we analyzed genes in adrenal glands including those modulating transcription via the D-box element, namely *Dbp* and *E4bp4* (Fig. 4). *Dbp* gene expression showed a clear circadian oscillation in both SHR and WKY animals, but the circadian phase was again advanced by ~ 4 h in SHR. Interestingly, *E4bp4* showed a relatively weak circadian oscillation in WKY rats compared with SHR. Furthermore, the peak amplitude of expression was markedly elevated in SHR and occurred in the dark period rather than the light.

Diurnal E4bp4 gene expression corresponds to Rora gene expression in the liver

E4bp4 and *Rora* gene expression in liver was subsequently analyzed (Fig. 5). Circadian oscillation of *Rora* gene expression was more clearly observed in SHR than WKY animals. In comparison with *Rora* gene expression by WKY controls, it was significantly higher at the middle of the dark period and lower at the middle-to-end of the light phase in SHR. Peak expression of the *E4bp4* gene was confirmed from the middle of the dark period to the beginning of the light phase in both SHR and WKY animals. *E4bp4* gene expression by SHR was significantly higher than in controls at ZT0.

Circadian rhythms of serum levels of corticosterone and aldosterone show phase advances in the SHR

Serum levels of corticosterone, the dominant glucocorticoid in rodents, and aldosterone in SHR and WKY animals were analyzed to evaluate circadian differences in adrenal gland
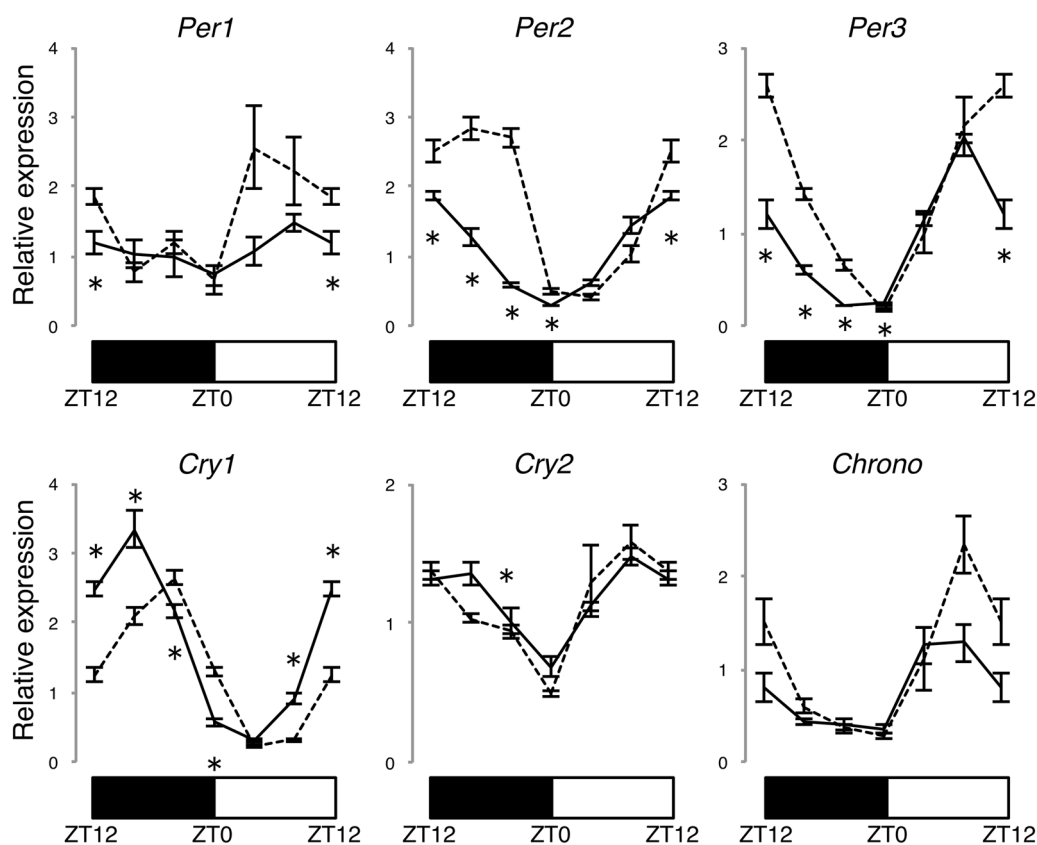
Fig. 2 Diurnal gene expression of *Per*, *Cry,* and *Chrono* in the adrenal gland. Diurnal expression patterns of *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2* and *Chrono* in the adrenal gland of SHR (solid line) and WKY (dashed line) rats. The horizontal bar indicates the time course: black indicates the 12 h dark period from ZT12 (2000 h) to ZT0 (0800 h); white indicates the 12 h light period from ZT0 (0800 h) to ZT12 (2000 h). The vertical axis indicates the relative gene expression levels normalized to *Gapdh*. The data represent the means ± standard error of the mean. ZT, Zeitgeber time. *$P < 0.05$

output (Fig. 6). The serum corticosterone levels of both rat strains tended to be higher during the light phase than in the dark. Although the difference of corticosterone levels in SHR and WKY animals at ZT did not achieve statistical significance, the circadian surge was observed earlier in SHR (ZT20 to ZT0) than in WKY rats (ZT0 to ZT4). Peak serum aldosterone was confirmed to occur later than corticosterone in both SHR and WKY animals. As with the difference in circadian rhythms for corticosterone in SHR and WKY rats, the circadian surge occurred earlier in SHR (ZT4 to ZT8) than in WKY rats (ZT8 to ZT12). This resulted in a significantly higher serum aldosterone level in SHR than WKY animals at ZT8.

Discussion

The present study analyzed the expression patterns of circadian clock genes and the *StAR* marker gene in the adrenal glands, and the circadian rhythms of serum corticosterone and aldosterone levels in SHR and control WKY rats. By mRNA expression analysis, we detected subtle differences in circadian gene expression in SHR and normotensive WKY control rats, most prominently, a phase advance in gene expression. Specifically, *Bmal1*, *Per2*, *Per3*, *Cry1*, *RevErba*, *Revervb,* and *Dbp* gene expression was phase advanced in SHR relative to WKY rats. An advanced phase of expression of clock genes that constitute the primary feedback loop (*Bmal1*, *Per2*, *Per3,* and *Cry1*) would be expected to affect the transcription of genes that carry an E-box element in their promoter, such as *Dbp*, *RevErba,* and *Revervb* [17].
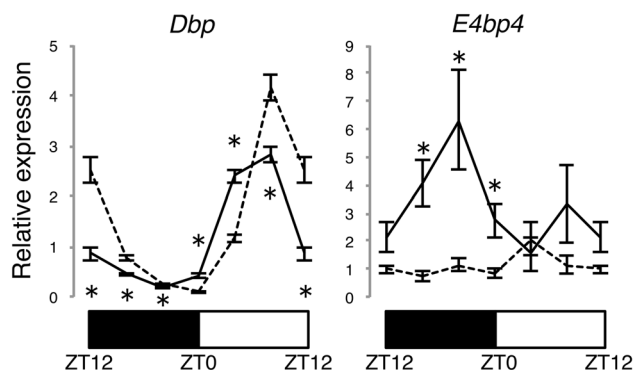
We also investigated the gene expression pattern of *StAR* in adrenal glands from SHR. *StAR* is another CLOCK–BMAL1-controlled gene and is a rate-limiting enzyme for glucocorticoid synthesis [5]. Here, we found that *StAR* peak expression was also advanced in SHR adrenal glands relative to WKY rats. These data support the notion that an advanced circadian clock influences the transcription of CCGs in SHR adrenal glands. This may result in an early surge of daily glucocorticoid secretion in SHR, thus propagating the phase advance of the adrenal circadian clock to other organs, as previously described [1, 5, 14–16]. Indeed, our present observational study revealed
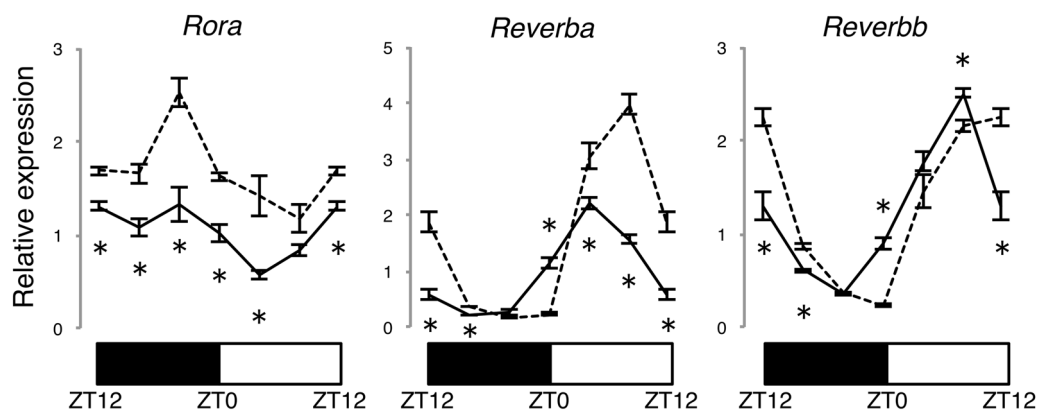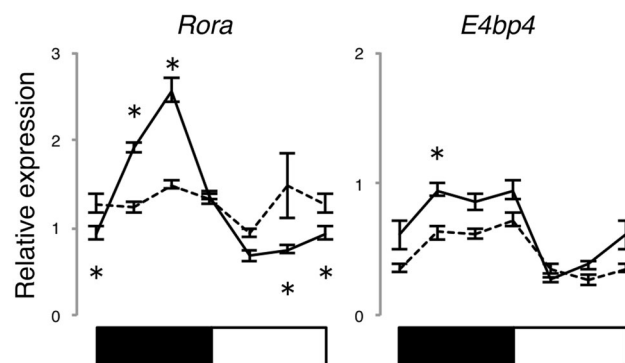
Fig. 3 Diurnal gene expression of *Rora*, *Reverba*, and *Reverbb* in the adrenal gland. Diurnal expression patterns of *Rora*, *Reverva*, and *Reverbb* in the adrenal gland of SHR (solid line) and WKY (dashed line) rats. The horizontal bar indicates the time course: black indicates the 12 h dark period from ZT12 (2000 h) to ZT0 (0800 h); white indicates the 12 h light period from ZT0 (0800 h) to ZT12 (2000 h). The vertical axis indicates the relative gene expression levels normalized to *Gapdh*. The data represent the means ± standard error of the mean. ZT, Zeitgeber time. *$P < 0.05$

Fig. 4 Diurnal gene expression of *Dbp* and *E4bp4* in the adrenal gland. Diurnal expression patterns of *Dbp* and *E4bp4* in the adrenal gland of SHR (solid line) and WKY (dashed line) rats. The horizontal bar indicates the time course: black indicates the 12 h dark period from ZT12 (2000 h) to ZT0 (0800 h); white indicates the 12 h light period from ZT0 (0800 h) to ZT12 (2000 h). The vertical axis indicates the relative gene expression levels normalized to *Gapdh*. The data represent the means ± standard error of the mean. ZT, Zeitgeber time. *$P < 0.05$


Fig. 5 Diurnal gene expression of *Rora* and *E4bp4* in the liver. Diurnal expression patterns of *Rora* and *E4bp4* in the liver of SHR (solid line) and WKY (dashed line) rats. The horizontal bar indicates the time course: black indicates the 12 h dark period from ZT12 (2000 h) to ZT0 (0800 h); white indicates the 12 h light period from ZT0 (0800 h) to ZT12 (2000 h). The vertical axis indicates the relative gene expression levels normalized to *Gapdh*. The data represent the means ± standard error of the mean. ZT, Zeitgeber time. *$P < 0.05$

advanced phase circadian rhythms for serum corticosterone and aldosterone levels in SHR. These results suggest that the core clock components in the adrenal glands of SHR run faster than in WKY controls. This influences the expression of CCGs and results in phase advance of biological output from the adrenal gland.

Regarding *E4bp4* gene expression, in a wild-type situation, RORA binds the *E4bp4* RRE sequence to activate *E4bp4* gene transcription; thereafter, translated E4BP4 binds the D-box sequence of *Rora*, *Reverba*, and *Reverbb* to suppress their transcription, thus forming a negative feedback loop. We found that *E4bp4* gene expression was significantly higher in SHR adrenal glands than in WKY rats, especially in the dark period, despite *Rora* gene expression remaining consistently low across a 24 h period in SHR.

Consequently, we propose that elevated *E4bp4* expression in SHR adrenal glands is independent of RORA activation. We also analyzed circadian rhythms of *Rora* and *E4bp4* gene expression in the liver, because previous research, in which the liver and the colon were analyzed, showed tissue specificity of peripheral circadian clock alterations in SHR [14]. In the liver, higher expression of the *E4bp4* gene at ZT0 was also confirmed, but this does not conflict with higher *Rora* gene expression during the dark period, unlike the findings for the adrenal gland.

Our final key observation was that *Per1*, *Per2*, *Per3*, *Reverba*, and *Reverbb* gene expression tended to be lower overall in SHR adrenal glands than WKY adrenal glands. The *Per* and *Reverb* genes all carry a D-box in their promoter. These findings may suggest, therefore, that the RORA-independent mechanism that elevates *E4bp4*
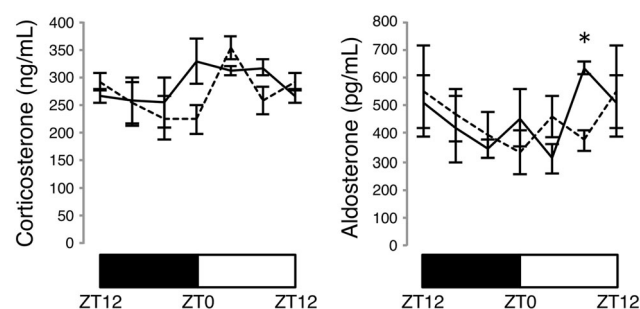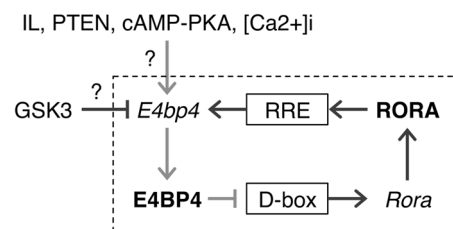
Fig. 6 Diurnal rhythms of serum corticosterone and aldosterone level. Diurnal rhythms of serum corticosterone and aldosterone level of SHR (solid line) and WKY (dashed line) rats. The horizontal bar indicates the time course: black indicates the 12 h dark period from ZT12 (2000 h) to ZT0 (0800 h); white indicates the 12 h light period from ZT0 (0800 h) to ZT12 (2000 h). The vertical axis indicates the serum level of corticosterone (ng/mL) and aldosterone (pg/mL). The data represent the means ± standard error of the mean. ZT, Zeitgeber time. $*P < 0.05$

Fig. 7 Hypothesized circadian feedback loop in the SHR adrenal gland. Transcriptional–translational relationships between *E4bp4* and *Rora* genes are shown by dashed squares. Red lines indicate strengthened processes, whereas blue lines indicate weakened processes. Arrows indicate activation, whereas flat-headed arrows indicate suppression/inhibition. RRE, rev response element; IL, interleukin; PTEN, phosphatase and tensin homolog; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; [Ca2 +]i, intracellular Ca2 + ; GSK3, glycogen synthase kinase-3

expression also causes transcriptional suppression of genes carrying a D-box sequence.

Molecular dissection of the circadian clock has been reported previously in several tissues of SHR and WKY rats [14, 15]. One study showed that 2-month-old SHR exhibited advanced circadian rhythms in their behavioral activity, clock gene and CCG expression in the SCN and colon in relative to WKY controls [14]. Another study reported an advanced circadian profile in *Clock* and *Bmal1* gene expression in the mediobasal hypothalamus (which anatomically includes the SCN) in SHR aged 8–10 weeks [15]. These reports, together with our current data, support the idea that SHR possess a circadian clock that runs faster than it does in WKY rats, in numerous tissues, including the adrenal gland.

A unique finding of our study is the marked high *E4bp4* gene expression in SHR adrenal glands. Previous studies that analyzed *E4bp4* gene expression in SHR found no differences in the liver and instead, found lower expression in the colon than in WKY rats [14]. *E4bp4* gene expression in the livers of SHR in our study was approximately the same as in that report, although it was higher than in WKY only at ZT0. Further, the circadian rhythm of *E4bp4* gene expression consistently reflected the rhythm of its activator gene *Rora*. These data imply that RORA-independent overexpression of *E4bp4* might be a tissue-specific circadian alteration affecting the adrenal gland of SHR. Although the primary cause underlying this phenomenon remains unknown, it may in turn give rise to low expression of CCGs carrying RRE or D-box and lead to aberrant biological output from the adrenal gland.

Recent studies proposed several mechanisms for induction of *E4bp4* transcription by pathways other than via the circadian clock feedback loop in different tissues, and these are candidates for mediating RORA-independent

overexpression of *E4bp4* (Fig. 7). Thus, certain interleukins (IL) were reported to be associated with *E4bp4* transcriptional activation, for example, its induction in B-cells by IL-3 and IL-4, explaining the association with B-cell survival and immunoglobulin E class switching, respectively [18, 19]. In T-cells, IL-27 induces *E4bp4* transcription, and this results in induction of *Tim-3* gene expression, IL-10 production, and reduction of gut inflammation [20]. Also in T-cells, phytohemagglutinin and tetradecanoyl phorbol acetate, or glycogen synthase kinase-3 inhibition, were reported to induce *E4bp4* transcription [21, 22]. Phosphatase and tensin homolog, a tumor suppressor, were shown to increase *E4bp4* transcription in endometrial cell lines [23]. In rats, parathyroid hormone-induced cAMP-PKA signaling activates *E4bp4* transcription in bone tissues [24, 25]. Also in rats, thapsigargin was reported to induce *E4bp4* transcription via intracellular Ca2 + concentration elevation in aortic smooth muscle cells [26]. Although these events are not directly reported to be associated with the SHR adrenal gland, they could be candidates involving high *E4bp4* gene expression. Overactivation of a G-protein-coupled receptor (GPCR) is one possible explanation, because cAMP-PKA signaling can mediate *E4bp4* gene expression [25]. SHR host a dysbiotic gut microbiota, which results in an imbalance of short-chain fatty acid production, which may act as ligands for corresponding tissue-specific GPCRs [27, 28]. As such, we propose that *E4bp4* gene expression might be due to adrenal gland-specific G-protein-coupled receptor activation. Probiotics may possibly improve circadian clock function in the adrenal gland, but future studies that aim to prove this hypothesis are required.

There have been some previous reports remarking on the relationship between the circadian clock and adrenal hormones in regulating blood pressure. In this context, one model is the *Cry*-null mouse, which is known to be hypertensive owing to aberrant circadian clock function. It exhibits salt-sensitive hypertension owing to D-box-

mediated overexpression of the *Hsd3b6* gene-encoding enzyme interplay at rate-limiting steps for aldosterone synthesis [8]. However, the developmental mechanism of this animal model does not explain the observations in the present study. First, the expression level of the *Dbp* gene which encodes the activator for D-box was decreased, and the level of expression of the *E4bp4* gene, which encodes a suppressor, was increased in the SHR adrenal gland. This would rather be expected to inactivate D-box-mediated transcription. Furthermore, serum aldosterone concentrations were not significantly higher in SHR than in WKY controls, although circadian phase advance was observed. On the other hand, it was shown that *Bmal1*-knockout mice exhibit a hypotensive phenotype with diminished diurnal variation in blood pressure and heart rates, and have significantly reduced levels of epinephrine, norepinephrine reflecting reduced expression of catecholamine-synthesizing cascade enzyme phenylethanolamine *N*-methyl transferase and monoamine oxidase B [7]. These results indicate BMAL1 regulates catecholamine synthesis positively. Therefore, the advanced circadian rhythm of *Bmal1* expression might lead to inappropriate circadian cycling of catecholamine synthesis, or lower expression of *Per1-3* might result in insufficient inhibition of CLOCK–BMAL1 activity and overactivation of catecholamine synthesis in SHR adrenal glands.

The present study does have some limitations. First, we analyzed only 5-week-old SHR in order to reduce circadian clock modification owing to acquired metabolic disorders in later life. There is a bidirectional influence between circadian clock and metabolic disorders. For instance, although clock mutations have been shown to relate to obesity, it has also been confirmed that acquired obesity can modify clock gene expression [29, 30]. Therefore, how the circadian clock changes in SHR with aging and the development of hypertension and insulin resistance is an important area, which should be investigated in future. Second, we did not investigate CCGs other than the *StAR* gene; therefore the impact of an altered circadian clock on adrenal function has not been exhaustively demonstrated. Further, the etiology of *E4bp4* gene overexpression remains unclear. Identifying these CCG expression and *E4bp4* gene regulating factors may lead to a new therapeutic target for hypertension relying on adrenal biological output. Finally, the design of this study was observational. Therefore, further research based on interventions influencing the circadian clock or factors reported to modify *E4bp4* gene expression should be performed.

In conclusion, our study has revealed that SHR possess an aberrant adrenal gland circadian clock, characterized by a phase advance in core clock gene and CCG expression, as well as RORA-independent overexpression of *E4bp4*.

Because the circadian rhythm of serum glucocorticoid levels was also phase advanced, circadian perturbations of the *StAR* transcriptional profile (reflecting the circadian clock) implies that there will be a biological impact of this aberrant circadian rhythm in the SHR adrenal gland.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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