

Chronopharmacological manipulation of Ang II-regulatable cell autonomous oscillators in adrenal zona glomerulosa cells

(副腎アルドステロン産生細胞におけるアンジオテンシンⅡ応答性
時計振動体の時間薬理的制御)

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Preface

The mammalian circadian timing system is organized in a hierarchy, with the master clock residing in the suprachiasmatic nucleus (SCN) of the hypothalamus and subsidiary peripheral clocks in peripheral tissues. The phase and rhythm of the clocks in peripheral tissues are influenced by various external and/or endogenous signals. A variety of neural, humoral, and metabolic factors, including glucocorticoids, insulin, glucose, and retinoic acid have been reported to affect circadian gene expression in cultured cell lines. External time signals such as feeding and physical activity also affect peripheral tissue clocks. It is also reported that several medically prescribed drugs (medical external inputs) drastically change clock gene expression. Because of the diversity of peripheral tissues and cell-types in the body, the existence of autonomous clock and identification of its potential entrainment signals, either external or internal, need to be empirically defined on a cell type-by-cell type basis.

In the present study, I focused on evaluating the basic circadian clock properties of adrenal zona glomerulosa cells. The zona glomerulosa, termed ZG, is a specific component of the adrenal gland cortex. The ZG cells are located in the outermost layer of the cortex and are responsible for synthesis and secretion of the steroid hormone aldosterone. As a critical element of the renin–angiotensin–aldosterone system (RAAS), these cells express the aldosterone-synthesizing enzyme (AS, or Cyp11b2) under the control of angiotensin II (Ang II) and play a role in blood pressure homeostasis. A previous study in our laboratory demonstrated that mice lacking the core clock genes *Cry1* and *Cry2* exhibit hyperplasia of ZG cells and resultantly show hypertension phenotype. Moreover, an additional study in our laboratory further demonstrated that Ang II has the ability to reset the phase of the autonomous circadian oscillator in ZG cells. However, it remains unclear how Ang II signaling is integrated into the molecular clock in ZG cells, and whether pharmacological blocking of Ang II signaling affects the phase of the ZG clock in vivo.

In this research background, my study in **Chapter 1** revealed that Ang II immediately upregulates mRNA expression levels of clock genes *PER1* and *E4BP4*, but not *PER2*, *CRY1*, *BMAL1*, and *DBP* in human adrenocortical H295R cells. In **Chapter 2**, I found that the AT1R blocker, CV11974 (CV, Candesartan), one of the most widely used anti-hypertensive drugs, mitigates Ang II-induced clock resetting in H295R cells. In **Chapter 3**, I identified potential *cis*-elements for Ang II-induced *PER1* and *E4BP4* transcriptional upregulation. In **Chapter 4**, I found that the time of CV administration affects the phase of the ZG rhythms in vivo.

Chapter 1: Ang II resets the clock through *PER1* and *E4BP4* induction in human H295R cells

The human adrenocortical H295R cells were treated with Ang II to test for the resetting effect of Ang II stimulation. Quantitative RT-PCR analysis revealed rhythmical expression of *PER1*, *PER2*, *BMAL1*, *CRY1*, *DBP*, and *E4BP4* that continued over 3 circadian cycles after Ang II treatment. There was an acute and transient upregulation of *PER1* and *E4BP4* mRNA expression immediately after Ang II stimulation. To facilitate realtime tracing of circadian clock gene expression, single- cell-level analysis of circadian rhythms was further performed by CCD video recording of H295R cells expressing luciferase reporter system. I observed that upon Ang II treatment, bioluminescence rhythms of individual cells were immediately resynchronized, suggesting that the immediate phase-resetting of individual cellular rhythms results in the emergence of overt rhythms at the cell population level.

Chapter 2: The AT1R blocker CV mitigates Ang II-induced clock resetting in H295R cells

CV is a receptor antagonist specific to Ang II type I receptor. I found that CV dose-dependently suppressed the Ang II-induced rhythm resetting and the amplitude of the rhythm triggered by Ang II stimulation in H295R cells. Furthermore, I observed that the magnitude of the initial induction of endogenous *PER1* and *E4BP4* mRNA expression in Ang II-treated cells was also decreased by CV in a dose-dependent manner. These data point to a previously unstudied potential action of the antihypertensive drug CV.

Chapter 3: Potential *cis*-elements for Ang II-induced *PER1* and *E4BP4* upregulation

I explored the potential *cis*-elements in their promoter and enhancer in the promoter and enhancer regions of *PER1* and *E4BP4*. Using reporter vectors containing tandem repeats of the putative *cis*-element with flanking regions, I showed that the human *PER1* CRE and *E4BP4* NBRE sequence significantly increased reporter transcription upon Ang II stimulation, which was effectively blocked by CV in a dose dependent manner. I thus propose that the *PER1* CRE and *E4BP4* NBRE sequence may be involved, at least partly, in the Ang II-regulated transcription of *PER1* and *E4BP4* in H295R cells.

Chapter 4: The effect of CV on the clock gene expression in ZG in vivo

I finally examined the effect of CV on the clock gene expression in ZG in vivo. By using laser microdissection, I found that CV administration downregulated mRNA expression of *Per1*, *E4bp4* and *Ngfib* in the adrenal ZG cells in mice. To evaluate the potential time-of-day-dependent effect of CV administration on the ZG clock in vivo, I administered CV to mice daily at a fixed time of day for seven consecutive days. While CV administration had no effect on locomotor activity rhythms, CV changed the ZG circadian clock phase in a dosing-time-dependent manner. These data of my CV experiments thus provide a critical potential example of how time-fixed medication can alter the phase of tissue clock. These data argue that drug-induced potential shifts in clock phase could influence target tissue homeostasis either positively or negatively, depending on the dosing time.

Based on the results from **Chapters 1–4**, I have, for the first time, experimentally demonstrated that Ang II signaling can act as external/internal cues to modulate the molecular clock and circadian rhythm in adrenal ZG cells. The results of my study strongly suggest that optimizing drug-dosing schedules (selecting optimal/appropriate timing) has the potential to avoid adverse potential side effect of drugs on target-tissue clock phase. Optimal timing may even restore or enhance local tissue clock function, thereby extending the scope of circadian clock research.

Introduction

Most aspects of physiology and behavior display circadian rhythms driven by an endogenous clock [1, 2]. In mammals, a master pacemaker in the suprachiasmatic nucleus (SCN) regulates downstream oscillators in peripheral tissues [3-5]. In all clock cell types, regardless of central vs peripheral, the basic clock system is composed of three elementary elements: an oscillator that oscillates even under constant conditions; an input that enables the oscillator to synchronize with environmental cycles or internal time cues; and an output that transmits the oscillator's signals into rhythmic gene expression and physiology, as referred to as the Eskinogram model [6]. Within the oscillator, opposing effects of transcriptional activators and repressors constitute interlocked feedback loops [7-9]. Briefly, the transcriptional activators CLOCK and BMAL1 drive transcription of *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) via binding to E-box elements present in their promoters. Once the repressor proteins PER and CRY reach a critical concentration, they form complexes and repress their own expression by inhibiting the CLOCK/BMAL1 complexes. In addition to E-box elements, the promoter regions of *Per* genes contain D-boxes, through which the PAR bZip family of transcriptional activators (DBP, TEF and HLF) and the related repressor E4BP4 exert their opposing effects on the expression of target genes [10-15]. The retinoic acid receptor-related orphan receptor binding elements (RRE) also provide a shared site for RORs (activator) and REV-ERBs (repressor), thereby forming a loop, in which REV-ERB α/β

proteins indirectly regulate expression of *Per* genes by suppressing the transcription of *Bmal1* and *E4bp4* through the RRE on their promoters [15-18]. In principle, phase entrainment or resetting of the clock must impact the activities or levels of the molecular components of these loops, and the induction of *Per1* gene expression is believed to be a critical step in this process [19-23]. Other parallel mechanisms that contribute to entrainment might exist. It would be expected that these unknown input pathways would also impinge on the loops described above.

The phase of the molecular clocks in peripheral tissues are influenced by various external and/or endogenous signals. Numerous endogenous neural, humoral, and metabolic factors, including glucocorticoids, insulin, glucose, retinoic acid, and Ang II have been reported to affect circadian gene expression in cultured cell lines [24-28]. External time signals such as feeding and physical activity also affect peripheral tissue clocks [29, 30]. It is also reported that several medically prescribed drugs (medical external inputs) drastically change clock gene expression [31-34]. Because of the diversity of peripheral tissues and cell-types in the body, the existence of autonomous clock and identification of its potential entrainment signal(s), either external (e.g., light, food intake) or internal (hormonal, neuronal, body temperature), need to be empirically defined on a cell type-by-cell type basis.

Our laboratory previously focused on evaluating the basic circadian clock properties of adrenal ZG cells. The zona glomerulosa, termed ZG, is a specific

component of the adrenal gland cortex. The ZG cells are located in the outermost zone of the cortex and responsible for synthesizing and secreting the steroid hormone aldosterone [35]. As a critical element of the renin–angiotensin–aldosterone system (RAAS), these cells express the aldosterone-synthesizing enzyme (AS, or Cyp11b2) under the control of Ang II and play a role in blood pressure homeostasis [36]. A previous study in our laboratory demonstrated that mice lacking the core clock genes *Cry1* and *Cry2* exhibit hyperplasia of ZG cells and resultantly show hypertension phenotype [37]. However, it is still not known (remains unexplored) whether ZG cells possess endogenous circadian oscillators, and if so, what their phase (time) entrainment signals are. This current situation contrasts to the extensive research on the circadian aspect of the adrenal gland in the hypothalamic-pituitary-adrenal (HPA) axis [38-41]. There are several adrenal-cortex *in situ* hybridization data in the literature, which suggest for the circadian gene expression in the region of ZG layer [38, 42]; however, direct evidence to show the rhythmicity of ZG cells is missing.

Within this paradigm, in our laboratory, the expression of clock genes within mouse adrenal ZG cells was firstly examined *in vivo*. ZG cells, lying in the outermost adrenocortical layer, were selectively isolated from the whole adrenal sections by using laser microdissection (Supplementary Fig. 1A). To pursue circadian changes driven by the endogenous clock, animals were kept in constant darkness and sampled the adrenals from them at 4-hour intervals

starting from circadian time (CT) 0 (the onset of the subjective morning). Quantitative RT-PCR analysis (Supplementary Fig. 1B, left) revealed that the expression levels of the key clock components *Period2* (*Per2*) and *Bmal1*—representatives of two major feedback loops within the clockwork—showed high-amplitude circadian oscillations with peak-to-trough ratios of ≈ 4.4 for *Per2* and ≈ 18.8 for *Bmal1*. A pronounced rhythmic expression was also observed for *Dbp*, a clock-controlled output gene. The peak phase of expression was detected around CT16 for *Per2*, CT20-0 for *Bmal1*, and CT8 for *Dbp*—a relationship (order) similar to that reported in peripheral tissues, such as in liver, kidney, and heart [43]. In addition to normal wild-type (WT) mice, clock-deficient mice were used to confirm that the observed rhythmicity originates from the molecular clock. In *Cry1*^{-/-}; *Cry2*^{-/-} (Supplementary Fig. 1B, middle) and *Clock* ^{$\Delta 19/\Delta 19$} (Supplementary Fig. 1B, right) mice, the rhythmic expression of *Per2*, *Bmal1* and *Dbp* was abolished. These data provide evidence that the rhythms in ZG cells are driven by the genetically encoded circadian clock.

Ex vivo adrenal tissue culture experiment was next conducted to test the self-sustainability of the rhythm in ZG cells. Time-lapse bioluminescence microscopy of isolated adrenal explants from *Per2*^{Luc} knock-in mice, which facilitate the analysis of circadian expression of *Per2* in tissues and cells [44], revealed abundant and robustly cycling *Per2*::Luc luminescence mostly in the outer layer of the cortex, in which ZG cells reside (Supplementary Fig. 2A), indicating continuation of circadian rhythms even without systemic cues. The

Per2::Luc rhythm was not detected in *Clock*^{Δ19/Δ19} adrenal slices (Supplementary Fig. 2A), which again indicate that the endogenous circadian clock mechanism drives this rhythm (see Supplementary Fig. 1B). Our laboratory next used dissociated cell culture of ZG cells (Supplementary Fig. 2B). For this particular purpose, *Per2-dluc* rat was used [45], since isolation of primary ZG cells has been established in rats [46]. Immunocytochemistry confirmed that approximately 90 percent of the cells isolated were immunolabelled for the ZG cell-marker Cyp11b2, an enzyme responsible for aldosterone production, verifying enrichment of ZG cells as reported [37] (Supplementary Fig. 2B). The freshly isolated rat ZG cells were maintained with repeated medium changes at 24-h intervals for 3 consecutive days (for establishment of primary cell culture) and subsequently maintained under constant culture conditions without medium change for an additional 96 h for luminescence tracing. Under these conditions, sustained circadian bioluminescence rhythms were observed (Supplementary Fig. 2B), indicating that cultured ZG cells can act as a circadian oscillator.

As an extension of the above investigation, our laboratory also asked whether the human adrenocortical cell line H295R exhibits circadian oscillation (Supplementary Fig. 2C). H295R cells have been widely utilized as a model system for studying ZG [47], including aldosterone biosynthesis [48, 49]. However, it is not known whether this human model cell line shares similar clock gene expression. Temperature is known to entrain (or synchronize) cellular

clocks of many tissues in vitro [50-52]. H295R cells were cultured for 3 days with a temperature cycle (12 h at 37°C, 12 h at 33°C) and placed them in constant temperature conditions (37°C). Cells were harvested every 4 h over a 48-h period to study circadian variations. Quantitative RT-PCR analysis (Supplementary Fig. 2C) revealed that the expression levels of *PER2* and *BMAL1*—representatives of two major feedback loops within the clockwork—showed high-amplitude circadian oscillations. *PER2* exhibited its circadian peak expression 4 h earlier than *BMAL1*, and *DBP* mRNA expression fluctuated in the opposite phase of *BMAL1*—a phasic relationship consistent with that observed in adrenal ZG cells in vivo (see Supplementary Fig. 1B) and the molecular model of the circadian clock [7-9]. These data indicate that mouse and rat ZG cells and human H295R cells all retain a functional clockwork.

Because Ang II is a potent physiological regulator of ZG function [35], our laboratory hypothesized that Ang II may modulate circadian rhythms in ZG. When Ang II was administered to cultured adrenal slices at different phases of luminescence, it caused phase-dependent phase-shifts (delay versus advance) (Supplementary Fig. 3A): administration at the luminescence peak (12 h after the trough) significantly delayed the phase, whereas administration 6 h after the peak advanced the phase significantly (Supplementary Fig. 3B).

Collectively, these data demonstrated that mouse ZG contains an Ang II-regulatable cell-autonomous circadian clock. However, it remains unclear how Ang II signaling is integrated into the molecular clock in ZG cells, and whether

pharmacological blocking of Ang II signaling affects the phase of the ZG clock in vivo. In this research background, using human adrenocortical H295R cells, I revealed that Ang II resets the clock through immediate upregulation of clock genes *PER1* and *E4BP4*, but not *PER2*, *CRY1*, *BMAL1*, *REV-ERB α* and *DBP* (**Chapter 1**). This clock resetting was mitigated by the Ang II type 1 receptor (AT1R) blocker CV11974 (CV, Candesartan), one of the most widely used anti-hypertensive drugs (**Chapter 2**). I further identified potential *cis*-elements for Ang II-induced *PER1* and *E4BP4* transcriptional upregulation (**Chapter 3**). Finally, I examined the effect of CV on the clock gene expression in ZG in vivo and found that the time of CV administration affects the phase of the ZG rhythms (**Chapter 4**).

METHODS

Animals

The C57BL/6J male mice 2–5 months old were used in this study. *Per2^{Luc}* knock-in mice [44] were bred and genotyped as described previously [37, 53–55]. Animals were housed under a regular 12-h light/12-h dark (LD) cycle, maintained at $22 \pm 2^\circ\text{C}$, with free access to food and water. For Figure 5, mice were placed on salt-deficient (0% NaCl) diets (OrientalBioService) and supplied with ion free water. CV11974 (Tocris Bioscience, 5 mg/kg, dissolved into 150 mM Tris-HCl [pH 7.4] buffer containing 0.25% DMSO) was injected intraperitoneally (i.p.) at either ZT10 or ZT22 after a one-week handling period. For sampling, animals were sacrificed by cervical dislocation at selected time points. Red safety lamps were used for sampling in the dark. All mouse experiments were conducted in compliance with the Ethical Regulations of Kyoto University and performed under protocols approved by the Animal Care and Experimentation Committee of Kyoto University.

Immunoblotting and quantitative RT-PCR (qRT-PCR)

H295R cells (ATCC, CRL-2128) were cultured in DMEM/F12 supplemented with 2.5% Nu serum (BD Biosciences) and 1% ITS premix (BD Biosciences) as described previously [54]. Cells were lysed in Laemmli buffer and immunoblotting was performed according to the standard method of our laboratory [56] with antibodies against NGFI-B (Santa Cruz, sc-5569, 1:500 dilution) or E4BP4 (Santa Cruz, sc-9549, 1:1000). For qRT-PCR, cells were

harvested in TRIzol reagent (Thermo Fisher). Total cell RNA was extracted using RNeasy micro kit (Qiagen) and converted to complementary DNA with SuperScript VILO cDNA synthesis kit (Thermo Fisher). Quantitative real-time PCR was achieved using the Platinum SYBR Green qPCR SuperMix-UDG with ROX (Thermo Fisher) or THUNDERBIRD SYBR qPCR mix (TOYOBO) with StepOnePlus system (Applied Biosystems) or by TaqMan qPCR analysis performed on a BioMark HD System (Fluidigm, South San Francisco, CA) using a 48.48 Fluidigm BioMark Dynamic Array chip (Fluidigm) [57]. The primer sets used in SYBR Green qPCR included: *Per1*, Fw: 5'-TGG CTC AAG TGG CAA TGA-3', Rv: 5'-GGC TCG AGC TGA CTG TTC ACT-3', *Per2*, Fw: 5'-AGA ACT ATC TCC AGG AGC GGT-3', Rv: 5'-CCG AGT CTT GAC GCG TTT TG-3', *Bmal1*, Fw: 5'-CCT AAT TCT CAG GGC AGC AGA T-3', Rv: 5'-TCC AGT CTT GGC ATC AAT GAG T-3', *Dbp*, Fw: 5'-AAT GAC CTT TGA ACC TGA TCC CGC T-3', Rv: 5'-GCT CCA GTA CTT CTC ATC CTT CTG T-3', *E4bp4*, Fw: 5'-CAC GGA CCA GGG AGC AGA AC-3', Rv: 5'-CGG ATG GAG GAG ACA AAT CAC-3', *Rplp0*, Fw: 5'-CTC ACT GAG ATT CGG GAT ATG-3', Rv: 5'-CTC CCA CCT TGT CTC CAG TC-3', *PER1*, Fw: 5'-GCA TCT CAG CGG AGC TCA CA-3', Rv: 5'-GAG GCT GTA GGC AAT GGA ACT G-3', *PER2*, Fw: 5'-GTG CAG CTC CAC CCT AGT GA-3', Rv: 5'-GAT TTT CCT GCT CCA TGG GTT GAT G-3', *CRY1*, Fw: 5'-AGC AAA CTC ACC TGT TGA AGC AAG G-3', Rv: 5'-GCT GCA ACA GTA TTC CTC CTG AAT G-3', *BMAL1*, Fw: 5'-AGT CTG TCT TCA AGA TCC TCA ACT AC-3', Rv: 5'-CTG GAA GTC CAG TTT TTG CAT CTA TG-3', *DBP*, Fw: 5'-GAA CCC GAC CCA GCT GAT CT-3', Rv: 5'-CTT GGC TGC CTC GTT GTT CTT GT-3', *E4BP4*, Fw: 5'-CCC GAG AGC AGG AAC ACG AT-3',

Rv: 5'-ACC CTA TCT ATG TGT GTA GGA GAA C-3', *REV-ERB α* , Fw: 5'-GGC AGG GCA ACT CAA AGA ATG TTC -3', Rv: 5'-GGT GAC TTG GTC ATG CTG AGA AAG-3', and *RPLP0*, Fw: 5'-ATG CAG CAG ATC CGC ATG T-3', Rv: 5'-TTG CGC ATC ATG GTG TTC TT-3'. TaqMan probe and primer sets were as follows; for *Per1*, probe, FAM-AGC CCC TGG CTG CCA TGG-TAMRA, Fw: 5'-CAG GCT TCG TGG ACT TGA GC-3', Rv: 5'-AGT GGT GTC GGC GAC CAG-3'; for *Per2*, probe, FAM-AGG CAC CTC CAA CAT GCA ACG AGC C-TAMRA, Fw: 5'-GCA CAT CTG GCA CAT CTC GG-3', Rv: 5'-TGG CAT CAC TGT TCT GAG TGT C-3'; for *Bmal1*, probe, FAM-CGC CAA AAT AGC TGT CGC CCT CTG ATC T-TAMRA, Fw: 5'-GTA CGT TTC TCG ACA CGC AAT AG-3', Rv: 5'-GTA CCT AGA AGT TCC TGT GGT AGA-3'; for *Dbp*, probe, FAM-CGG CTC CCA GAG TGG CCC GC-TAMRA, Fw: 5'-CGG CTC TTG CAG CTC CTC-3', Rv: 5'-GTG TCC CTA GAT GTC AAG CCT G-3'; for *E4bp4*, probe, FAM-CAG GGA GCA GAA CCA CGA TAA CCC ATG A-TAMRA, Fw: 5'-CGC CAG CCC GGT TAC AG-3', Rv: 5'-CAT CCA TCA ATG GGT CCT TCT G-3'; for *Rplp0*, probe, FAM-TGG CAA TCC CTG ACG CAC CGC C-TAMRA, Fw: 5'-GCG TCC TCG TTG GAG TGA C-3', Rv: 5'-AAG TAG TTG GAC TTC CAG GTC G-3'. The data were normalized to a non-oscillatory housekeeping gene, *RPLP0* mRNA levels.

AAV-mediated circadian clock reporter assay

A luciferase reporter (Luc2P) driven by a mouse or human *Per2* promoter sequence (positions -1670 to +53 for mouse; -1840 to +108 for human) was inserted between the inverted terminal repeat (ITR) sequences in pAAV-MCS2

plasmid (Addgene, Plasmid #46954) to obtain pAAV-*mPer2*-Luc2P or pAAV-*hPER2*-Luc2P. Recombinant AAV-DJ vectors were produced with a triple-transfection helper-free method as described [58]. The day after AAV transduction, cells were split and equally plated into 35-mm dishes containing medium supplemented with 1 mM D-luciferin.

Transcriptional enhancer reporter assay

Cells were transfected with the following reporter plasmids using Lipofectamine LTX (Thermo Fisher Scientific): (i) pGL4.25 *PER1* CRE×3-WT-Luc2CP, in which a tandem repeat of the sequence corresponding to the human *PER1* CRE with flanking sequences (positions –1950 to –1921; 5'-TTC TTC CGC TTT GAC GTC ACT GCT GTC TCC-3') was cloned into [luc2CP/ minP] (Promega), (ii) pGL4.25 *PRE1* CRE×3-Mut-Luc2CP, which is the same as (i) except that CRE sequences were mutated to 5'-TCACATAA-3', (iii) pGL4.25 *E4BP4* NBRE-like×3-WT-Luc2CP, in which a tandem repeat of the sequence corresponding to the human *E4BP4* NBRE-like (5'-TGACCTTG-3') with flanking sequences (positions +4452 to +4481) was cloned into pGL4.25, (iv) pGL4.25 *E4BP4* NBRE×3-WT-Luc2CP, in which a tandem repeat of the human *E4BP4* NBRE (5'-TGACCTTT-3') with flanking sequences (positions +4845 to +4874) was cloned into pGL4.25, and (v) pGL4.25 *E4BP4* NBRE×3-Mut-Luc2CP, which is the same as (iv) except that the human *E4BP4* NBRE sequences were mutated to 5'-TGAATTCT-3'. Bioluminescence recording was performed a day after transfection.

Bioluminescence recordings from cultured cells

Bioluminescence recording was performed using a dish-type luminometer AB-2550 Kronos Dio (ATTO) under 5% CO₂ atmosphere at 37°C. Luminescence was measured at 20-min intervals, with an exception of 30-min intervals in pharmacological experiments (due to drug application during intervals between measurements). To evaluate dose dependency, CV11974 was administrated at a concentration of 0.001, 0.01, 0.1, and 1 µM. Where indicated, cells were treated with Ang II (100 nM), CV11974 (1 µM) or PD123319 (1 µM, Abcam). For enhancer activity reporter assay, values were normalized to the luminescence intensity that was recorded 30 min before Ang II stimulation. For single cell level recording, successive images were acquired with a highly sensitive cryogenic CCD camera (600 series, Spectral Instruments) with a 20 min exposure time for each picture (3 pictures/h). A median filter was applied to all images to eliminate cosmic-ray-induced background noise.

Laser Microdissection

Cryosections of fresh frozen adrenal gland (7-µm thickness) were prepared using a cryostat microtome (CM1950, Leica) and mounted on 2 µm PEN membrane glass slides (Leica). Sections were fixed with ice-cold mixture of ethanol and acetic acid (19:1) and stained with 0.1% Cresyl Echt Violet. ZG were then excised using a LMD7000 device (Leica) under RNase-free conditions and lysed into TRIzol reagent (Invitrogen).

In situ hybridization

Radioisotopic in situ hybridization was performed with following gene-specific probes: for *Agtr1a*, nucleotides 1387–2138 (GenBank, NM_177322); for *Agtr1b*, nucleotides 1298–2015 (NM_175086); for *Cyp11b2*, nucleotides 1744–2456 (S85260). The corresponding cDNA fragment was cloned and used as a template for the generation of riboprobes. The riboprobes were radiolabeled with [³³P]UTP (PerkinElmer), using a standard protocol for the cRNA synthesis. In situ hybridization was performed as described [37]. Briefly, paraformaldehyde fixed tissues were frozen and sectioned at a thickness of 30 µm. Then, the free-floating tissue sections were transferred through 4 x SSC, proteinase K (1 µg/ml, 0.1 M Tris-HCl [pH 8.0]; 50 mM EDTA) for 15 min at 37°C, 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, and 4 x SSC for 10 min. The sections were then incubated in the Denhardt's hybridization buffer containing 55% formamide, 10% dextran sulfate, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.6 M NaCl, 0.2% N-laurylsarcosine, 500 µg/ml tRNA, 0.25% SDS, 10 mM dithiothreitol (DTT) and radiolabeled riboprobes for 16 h at 60°C. Following a high-stringency posthybridization wash, the sections were treated with RNase A. Air-dried sections were exposed to X-ray films (Kodak Biomax).

Data and statistical analysis

All statistical analyses were performed using GraphPad Prism 8, using the statistical tests for each figure as indicated in the legend.

RESULTS

Chapter 1: Ang II resets the clock through *PER1* and *E4BP4*

induction in human H295R cells

The human adrenocortical H295R cells were treated with Ang II to test for the resetting effect of Ang II stimulation (Fig. 1). To do this, H295R cells were seeded onto 24-well plates and cultured for 2 days to confluency; then, after Ang II treatment, cells were harvested at 0, 2, and every 4 hours over a 68-h period ($n = 3$ replicates; total 57 wells, Fig. 1). Quantitative RT-PCR analysis revealed rhythmic expression of *PER1*, *PER2*, *BMAL1*, *CRY1*, *DBP*, *REV-ERB α* , and *E4BP4* that continued over 3 circadian cycles after Ang II treatment. There was an acute and transient upregulation of *PER1* mRNA expression immediately after Ang II stimulation. In addition, a similarly prominent (~6 fold) upregulation of mRNA expression was also identified for *E4BP4*. These observations, taken together, indicate that Ang II stimulation has the ability to reset or (re)initiate circadian clock gene expression in H295R cells.

In order to facilitate realtime tracing of circadian clock gene expression, I next employed a luciferase reporter system. I generated adeno-associated virus (AAV) encoding a luciferase under the *PER2* promotor from either human or mouse origin and transduced it to H295R cells. Then, luminescence was traced both before and after Ang II stimulation, in culture (Fig. 2A). Luminescence rhythmicity gradually dampened before Ang II treatment. Following Ang II administration, a significant and high-amplitude surge was observed, along with

sustained luminescence oscillations in subsequent cycles ($P < 0.0001$, for the first surge and second circadian cycle, vs vehicle control, Fig. 2A). Single-cell-level analysis of circadian rhythms was further performed by CCD video recording of H295R cells before and after Ang II treatment (Fig. 2B). I observed that upon Ang II treatment, bio-luminescence rhythms of individual cells were immediately resynchronized. This was evident as the vertical alignment of peak bioluminescence across all traced cells in the heatmap of cellular rhythms after Ang II treatment. These data illustrate the phase-resetting of individual cells (see also ref. [59]).

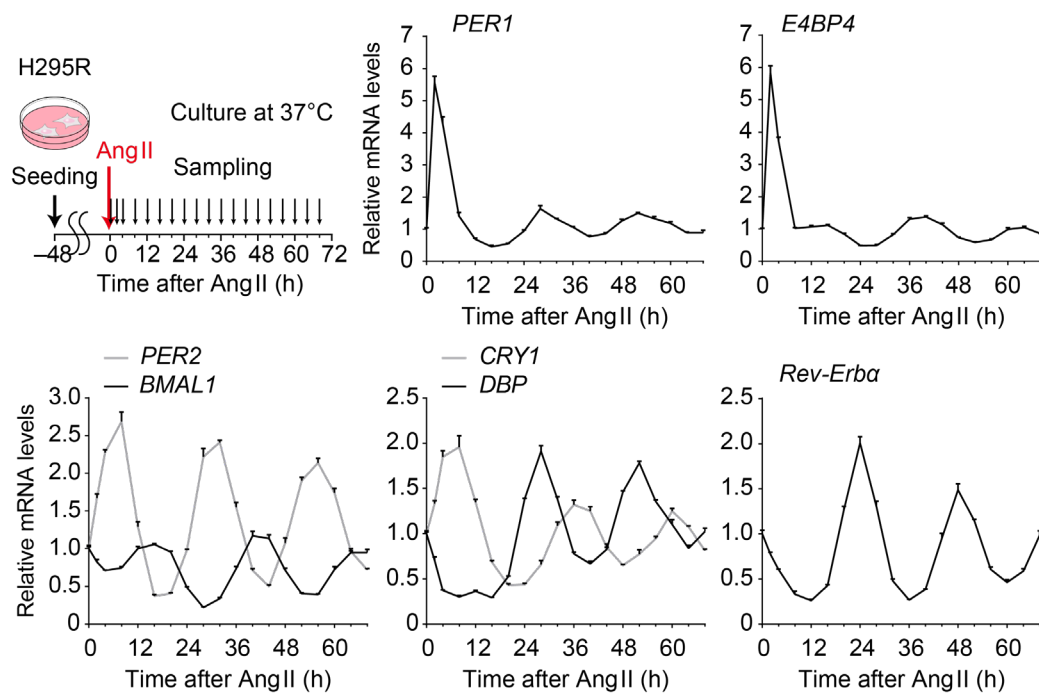


Fig. 1 | Circadian expression profiles of representative core clock genes and clock-controlled genes in H295R cells. Cells were treated with Ang II at Time 0 and were harvested at 0, 2, and every 4 hour over a 68-h period. Values are means $\pm$ s.e.m. from $n = 3$ biological replicates per timepoint.

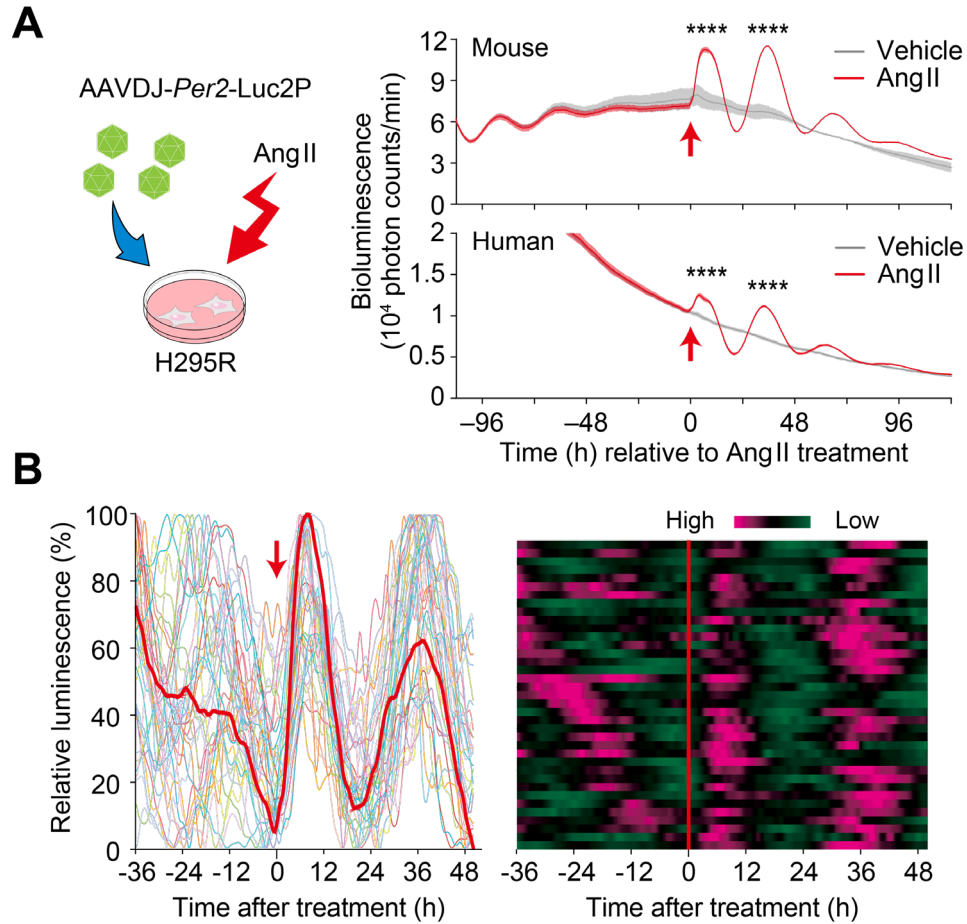


Fig. 2 | Ang II has the ability to reset circadian oscillation of H295R cells. (A) A cartoon for viral infection to H295R cells and luminescence traces of cells harboring a luciferase reporter under the control of human or mouse *Per2* promoter. Arrows indicate the time of Ang II or vehicle administration. Data are the means $\pm$ s.d. from $n = 4$ biologically independent traces. **** $P < 0.0001$, unpaired two-sided Student's *t* test. **(B)** Single-cell bioluminescence tracing in H295R cells expressing mouse *Per2*-Luc reporter. Heat maps show individual cellular luminescence, where magenta corresponds to peak bioluminescence and green to trough.

Chapter 2: The AT1R blocker CV mitigates Ang II-induced clock resetting in H295R cells

Because CV, one of the most commonly used antihypertensive drugs, is a receptor antagonist specific to AT1R, I examined whether CV treatment suppressed the rhythm resetting induced by Ang II. Using the H295R cells virally transfected with *Per2-Luc* reporter, I observed that Ang II-induced *Per2-Luc* expression declined with increasing CV doses (0, 1, 10, 100, and 1000 nM, Fig. 3A). This resulted in a similar dose-dependent attenuation of the *Per2-Luc* rhythm amplitude in the following circadian cycles (Fig. 3A). Moreover, CV shortened the duration of the initial surge of the *Per2-Luc* expression, which in turn influenced the phase of the *Per2-Luc* rhythms in the subsequent circadian cycles (Fig. 3B; compare phases across different CV doses). I observed that the Ang II type 2 receptor inhibitor PD did not affect either the amplitude or phase of the *Per2-Luc* rhythm in the same H295R cells (Supplementary Fig. 4).

Furthermore, I observed that the magnitude of the initial induction of endogenous *PER1* and *E4BP4* mRNA expression in Ang II-treated H295R cells was also decreased by CV treatment in a dose-dependent manner (Fig. 3C). Considering that CV is one of the most commonly used clinical drugs for hypertension treatment, its dose-dependent effects on clock gene expression imply a previously unstudied potential action of the drug (for known actions of CV, see [60]).

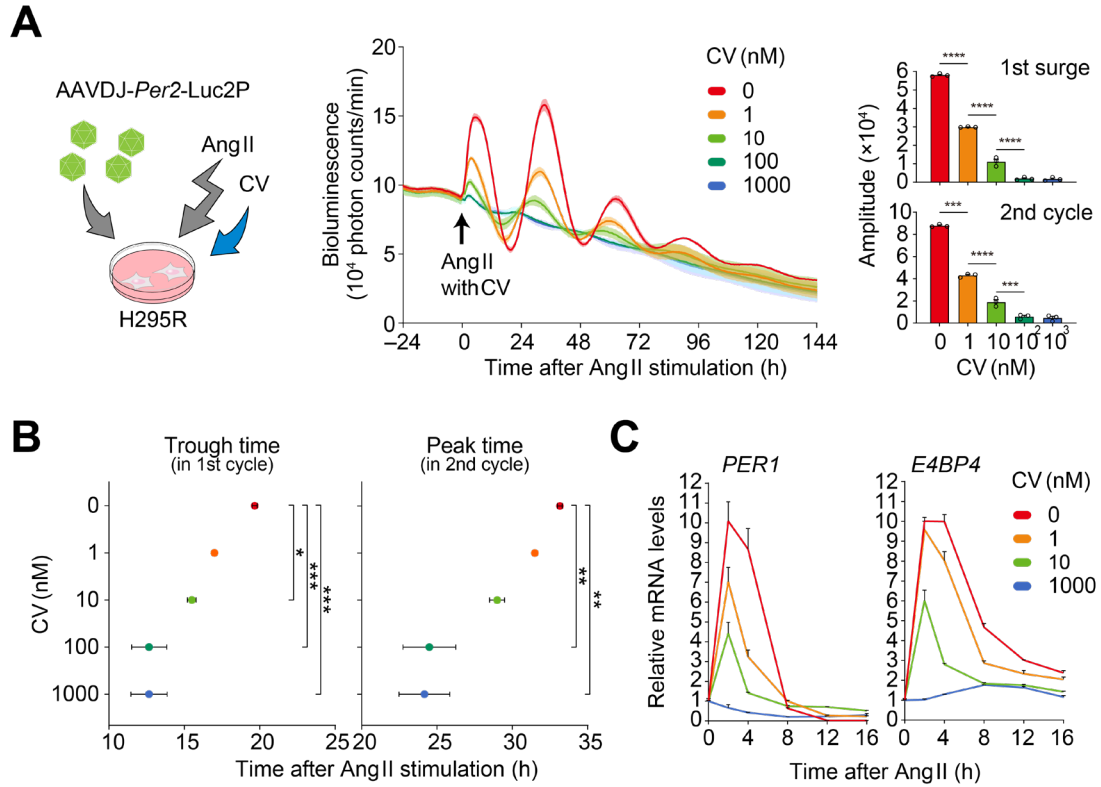


Fig. 3 | The AT1R blocker dose-dependently mitigates Ang II-induced clock resetting in H295R cells. (A-B) Effects of increasing concentrations of CV on Ang II-induced circadian luminescence in H295R cells. Cells were transduced with a luciferase reporter under the control of mouse *Per2* promoter. Arrows indicate the time of Ang II/CV treatment. Bar graphs in (A) illustrate the amplitude of the first surge and second cycle of luminescence following Ang II administration with different doses of CV. Plots in (B) show the first trough and second peak phase of the luminescence rhythm following Ang II treatment. $n = 3$ biological replicates for each CV concentration. Traces in (A) are expressed as means $\pm$ s.d. **(C)** Effects of CV on Ang II-induced *PER1* and *E4BP4* mRNA expression in H295R cells. $n = 3$ biological replicates per datapoint. Values are means $\pm$ s.e.m. Statistics in (A), (B) were one-way ANOVA followed by Tukey's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Chapter 3: Potential *cis*-elements for Ang II-induced *PER1* and *E4BP4* upregulation

It has been reported that *PER1* is acutely responsive to various stimuli through the cAMP-response element (CRE) located in its promoter and that Ang II causes upregulation of CRE-mediated gene transcription in H295R cells [61-66]. Indeed, the isolated human *PER1* CRE sequence significantly increased reporter transcription upon Ang II stimulation, and CV treatment dose-dependently reduced this activation (Supplementary Fig. 5A–C). However, CRE has not previously been implicated in the regulation of *E4BP4*. Given this, I propose that the NGFI-B responsive element (NBRE, 5'-TGACCTTT-3') located in intron 1 (Fig. 4A) may play a role in the observed Ang II/CV-dependent mRNA induction of *E4BP4* (Fig. 4A–D). *E4BP4* consists of two exons. Sequence conservation analysis revealed that the intron 1 contains a highly conserved segment (+4283 to +5181, relative to the transcription start site) harboring two putative NBRE sequences at +4459 to +4466 (NBRE-like) and +4852 to +4859 (NBRE-consensus), but no CRE sites (see Fig. 4A). Western blots (Fig. 4B) confirmed acute accumulation of NGFI-B, noticeable as early as 2 h after Ang II treatment, consistent with previous reports [48, 56]. *E4BP4* protein levels peaked around 8 h after Ang II treatment (Fig. 4B). Subsequently, I assessed the potential *cis*-element function using reporter vectors containing tandem repeats of the putative *cis*-element with flanking regions (NBRE-consensus×3-luc and NBRE-like×3-luc) (Fig. 4C). In H295R cells, Ang II significantly increased the

reporter activity of NBRE-consensus, and CV effectively blocked this effect. Contrastingly, neither Ang II nor CV had any effect on the NBRE-like sequence, indicating the sequence specificity of Ang II-induced transactivation in H295R cells. I confirmed that mutation of the NBRE-consensus sequence abolished its Ang II-dependent enhancer activity (Fig. 4C, Mut). Finally, I applied CV at the same various doses as used in Fig. 3A. I found that the upregulation of NBRE-consensus reporter activity was dose-dependently reduced by CV, in a manner similar to the changes observed in endogenous *E4BP4* mRNA expression in H295R cells (Fig. 4D, see also Fig. 3C). I thus propose that the NBRE sequence in intron 1 may be involved, at least partly, in the Ang II-regulated transcription of *E4BP4* in H295R cells, and CV may counteract the Ang II-induced phase-resetting by interfering with this transcriptional regulatory mechanism.

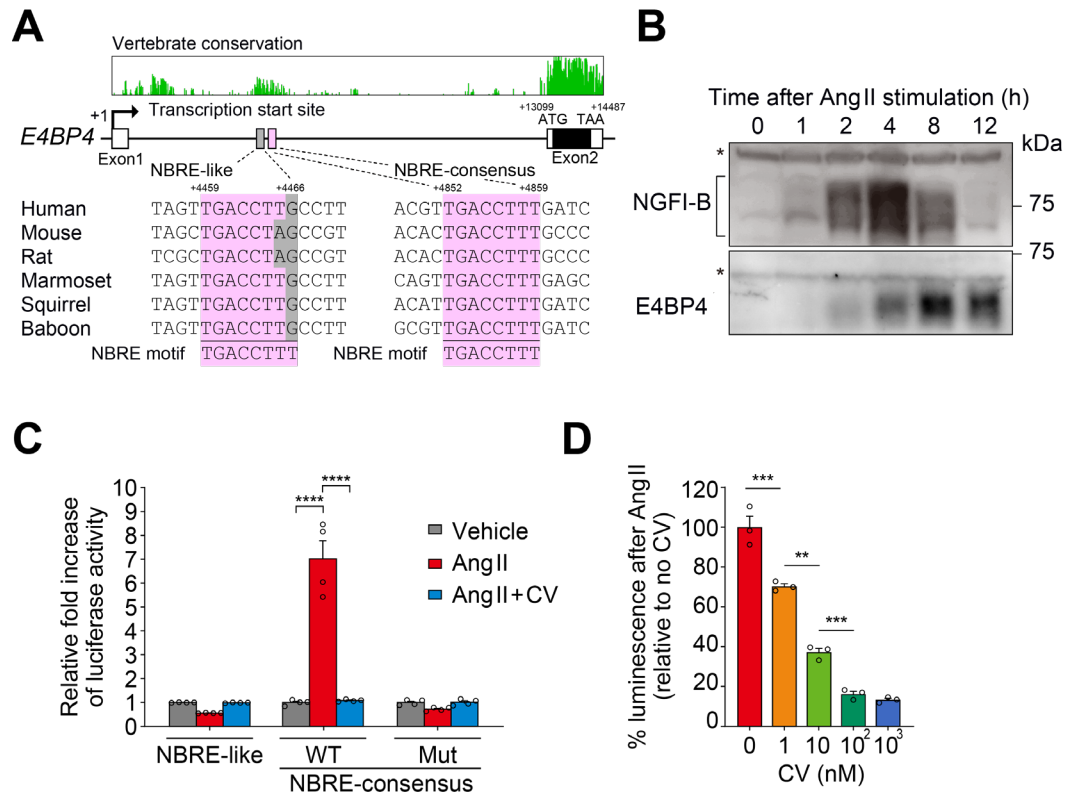


Fig. 4 | Putative *cis*-regulatory element involving Ang II-induced upregulation of *E4BP4* mRNA expression.

(A) Sequence alignment of two candidate NBREs, both located in the intron 1 of *E4BP4*. The sequences of NBRE-like (*left*) and NBRE-consensus (*right*) are aligned among species with the consensus NGFI-B binding motif (5'-TGACCTTT-3' or 5'-AAAGGTCA-3'). *E4BP4* consists of two exons. **(B)** Immunoblots showing the protein expression profiles of NGFI-B and *E4BP4* after Ang II stimulation. **(C)** Relative reporter activities of NBRE-like $\times$ 3-luc, NBRE-consensus $\times$ 3-luc, and its mutant (Mut, TGAATTCT). $n = 4$ biological replicates. **(D)** Dose-dependent effect of CV on NBRE-consensus reporter activity after Ang II stimulation. $n = 3$ biological replicates for each CV concentration. Statistics in (C) were two-way ANOVA followed by Sidak's multiple comparison test; in (D), one-way ANOVA followed by Tukey's multiple comparisons test. Values are means $\pm$ s.e.m.; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Chapter 4: The effect of CV on the clock gene expression in ZG in vivo

Because CV altered the phase and amplitude of the rhythms in ZG cells in vitro, I finally examined the effect of CV on the clock gene expression in ZG in vivo. A single bolus of CV was administered intraperitoneally either at ZT10 (2 h before active phase) or ZT22 (2 h before resting phase) following a one-week handling period (Fig. 5A, handling was performed in order to reduce the effect of stress caused by drug injection). ZG tissue was harvested via laser microdissection 4 h after CV administration, and then the mRNA expression levels of clock genes and *Ngfib* were analyzed by qRT-PCR. A single dosage of CV acutely suppressed the mRNA expression of *Per1*, *Ngfib* and *E4bp4*, all of which are Ang II-responsive genes (Fig. 5A, see also Supplementary Fig. 1). Notably, the expression of *Ngfib* and *E4bp4* mRNA expression was downregulated regardless of the time of CV administration, while *Per1* expression level was altered only when CV was administered at ZT22. On the other hand, CV had no effect on the mRNA expression of Ang II-nonresponsive genes including *Per2*, *Bmal1*, and *Dbp* (Fig. 5A, see also Supplementary Fig. 1).

Considering the routine use of CV as a daily treatment over long periods in clinical settings, I investigated whether the timing of daily CV administration influences the phase of the ZG clock. Mice received CV intraperitoneally at either ZT10 or ZT22 for 7 consecutive days, and ZG tissue was sampled every 4 h over a 24-h period. I found that the ZG clock phase was significantly altered in a

dosing-time-dependent manner (Fig. 5B, bottom). In mice receiving daily CV administration at ZT10, peak expression of clock genes in the ZG was observed at ZT10 for *Per1*, ZT14 for *Per2*, ZT22 for *Bmal1*, ZT6 for *Dbp*, and ZT18 for *E4BP4*, which is almost identical to the peak expression time observed in healthy mice (see Supplementary Fig. 1). However, daily CV administration at ZT22 resulted in a ~12-h phase shift, with peaks occurring at ZT2 for *Per1* and *Per2*, ZT6 for *Bmal1*, ZT18 for *Dbp*, and ZT22 for *E4BP4* (Fig. 5B). In contrast, circadian behavioral rhythms remained unaffected in CV-treated mice regardless of the timing of CV administration, underscoring CV's specific effect on the ZG (Fig. 5B, upper right). Using in situ hybridization with radioisotope-labeled probes, I verified the expression of AT1R subtypes *Agtr1a* and *Agtr1b* in the mouse ZG (Supplementary Fig. 6). These findings suggest that the timing of AT1R blocker administration significantly impacts the ZG clock phase in vivo.

Discussion

In the present study, using the ZG-related human adrenocortical cell line H295R, I found that Ang II stimulation reset the clock along with upregulation of *PER1* and *E4BP4* mRNA expression. Ang II type I receptor specific blocker CV, a widely used drug for hypertension treatment, dose-dependently suppressed the phase resetting and modulated the clock phase in H295R cells. The magnitude of the initial induction of *PER1* and *E4BP4* by Ang II treatment was also decreased by CV treatment in a concentration-dependent manner. In vivo, I showed that the circadian clock phase in the ZG is affected by the time of CV administration. Overall, these data demonstrate that Ang II and its receptor blocker CV act as internal/external cues to modulate the molecular clock and circadian rhythm in adrenal ZG cells, possibly through a mechanism involving gene expression of *PER1* and *E4BP4* (Fig. 6, model).

In this study, I found that *E4BP4* mRNA and protein levels were robustly and rapidly elevated by Ang II in H295R cells. *E4bp4* (also known as *Nfil3*) has been recognized as a key regulator in the field of immune system and neurobiology, as well as in the circadian clock biology. In CD4⁺ T cells, *E4bp4* is upregulated by chronic antigen stimulation and mediates upregulation of production of IL-10 and IL-13, which helps prevent excessive immune responses [67]. In natural killer (NK) cells, *E4bp4* activation enhances the cytotoxic activity, contributing to cancer suppression [68, 69]. In epilepsy, reduced expression of *E4bp4* in neurons causes an increased incidence of seizure [70]. *E4bp4* activation in microglia restrains microglial cell activation via ERK1/2 signaling

and alleviates delirium-associated cognitive decline [71]. In terms of circadian biology, on the other hand, the direct functional role of *E4bp4* is still not fully understood [72-74]; knockout studies show that *E4bp4* is dispensable for maintaining circadian rhythms of mouse locomotor activity [72, 74], while knockdown of *E4bp4* lengthened the circadian period in cultured rat-1 cells [73], indicating that *E4bp4* is not an essential requirement of the central clock function. Nevertheless, it is still possible that *E4bp4* may contribute to peripheral clock time-keeping and/or time-resetting system of certain tissue/cell types. Yoshitane *et al.* demonstrated that in in vitro cultured mouse embryonic fibroblast cells, acidic stress (pH 7.0 → pH 6.6) caused acute upregulation of *E4bp4* mRNA-protein expression and showed that genetic deficiency of *E4bp4* resulted in abrogated phase-resetting due to acid stress in the cells [74], indicating the potential of *E4bp4* as a phase-modulator for peripheral cells. In the current study, I observed that *E4BP4* mRNA-protein expression is strongly and acutely upregulated by Ang II to the levels almost comparable to those of acute induction of *PER1* in H295R cells (Fig. 1). *PER1* induction has long been considered to be involved in phase resetting of cells [19-23]; however, remaining phase-resetting capacities of *PER1* knockout cells (U2OS cells) to several resetting stimuli in culture (serum shock and forskolin) [75] and the fact that grafting *Per1* knockout embryonic fibroblasts into wild-type mice results in synchronization with the host's rhythm in vivo [76] support the idea that *Per1* induction is not the sole control point for resetting the clock and rather suggest that the responsible mechanism relies on a broader network of changes beyond

Per1 induction [74, 77]. In this light, exploration of the extent to which *E4BP4* contributes to Ang II-induced clock resetting will be a subject of a future study [12, 78]; additional investigations using *PER1* and *E4BP4* double deficient cells and knockout mice will be required to assess the mechanism of Ang II-induced molecular clock resetting.

NGFI-B is a potential transcriptional factor responsible for the Ang II-induced *E4BP4* NBRE activation. Our laboratory previously characterized NGFI-B as a NBRE-binding protein that mediates Ang II-induced *CYP11B2* and *HSD3B1* mRNA expression [48]. Consistent with this, Ang II caused protein accumulation of NGFI-B whose peak was observed at 4 h after Ang II administration; the peak of protein accumulation of *E4BP4* was later than NGFI-B (~8 h) (Fig. 4B), supporting our hypothesis that Ang II-induced *E4BP4* expression is under the regulation of NGFI-B. Nevertheless, there is currently no experimental evidence establishing a causal relationship between *E4BP4* NBRE activation and Ang II-induced NGFI-B expression. Further investigations including genetic deletion/mutation studies of *E4BP4* NBRE or chromatin Immunoprecipitation assay of the *E4BP4* enhancer are needed to demonstrate the role of NGFI-B on *E4BP4* transcriptional regulation.

The Ang II type 1 receptors have been localized in vascular smooth muscles, adrenal gland, kidney, heart, brain, platelets, and placenta [79]. Previous in vitro studies with cultured cells indicate that Ang II signaling through AT1R has the ability to phase reset the clock gene expression in cardiomyocytes and vascular smooth muscle cells [28, 80]. However, with regard to in vivo

relevance, no previous studies demonstrate the effect of endogenous Ang II on circadian clock function in peripheral tissues. Particularly, it is still unclear whether Ang II functions as an endogenous entrainment signal for peripheral tissues such as the ZG cells I identified in this study. In this regard, it is noteworthy that a modest diurnal rhythm of plasma Ang II concentration was reported for humans and rats [81-85] while plasma angiotensinogen levels do not exhibit significant circadian oscillation [86]. Thus, in vivo role of Ang II as a systemic entrainer remains to be studied in the future research. Clinically, Ang II itself is not used for therapeutic purposes. By contrast, CV is one of the most widely prescribed drugs for clinical treatment of hypertension and related cardiovascular diseases such as heart failure, myocardial infarction and diabetic nephropathy [87-91]. Thus, exogenously given (or taken) CV may rather warrant attention from a practice point of view.

In the present study, I found that the time of clinically used drug CV treatment remarkably influenced circadian phase in ZG cells both in vitro and in vivo. Repeated CV administration at ZT22 shifted the peak expression of clock genes in the ZG by ~12 h compared to non-treated conditions, whereas CV treatment at ZT10 had little effect on the ZG rhythms (see Fig. 5B and Supplementary Fig. 1). In contrast, circadian rhythm of locomotor activity remained unchanged regardless of the timing of CV administration (Fig. 5B). These findings suggest that CV dosing at ZT22 (bedtime of mice) could cause desynchronization between the ZG clock and the master clock in the SCN. Internal desynchronization has been associated with a range of negative mental

and physical health outcomes [2, 92]. Therefore, the time of CV administration should need to be carefully considered to minimize potential side effects arising from shifts in the ZG clock phase. Alternatively, optimizing the dosing schedules may have a positive effect on restoring or enhancing local clock function and improving tissue homeostasis. Overall, strategically timing medication has the potential to optimize therapeutic outcomes and minimize adverse effects.

In humans, bedtime treatment with AT1R blockers including CV results in a greater therapeutic effect on asleep blood pressure, reduced prevalence of non-dipping, and lower incidence of cardiovascular disease events [93, 94]. These beneficial effects of bedtime CV treatment in human contrast with my observations in mice, potentially due to differences in nocturnal (mice) versus diurnal (humans) activity patterns. Further studies using diurnal rodent models or primates may help clarify the relationship between CV's effects on the ZG clock and its therapeutic outcomes.

It has been reported that dosing time-dependence of drug effects is attributed to circadian changes in drug pharmacokinetics and pharmacodynamics [95]. Circadian changes in drug pharmacokinetics are thought to partially account for the dosing-time effect of CV; plasma AT1R blocker concentration in orally administered rats have been reported to show diurnal fluctuations, which is dominantly generated by the circadian drug absorption rhythms in the intestine [96]. To avoid the effect of dosing time-dependent changes in pharmacokinetics, in this study, I administered AT1R

blocker to mice intraperitoneally. Even under these conditions, I found that daily administration of AT1R blocker did affect ZG circadian oscillators in a dosing time-dependent manner (Fig. 5B). Further investigation is needed to clarify the precise mechanisms accounting for the time-of-day-dependent pharmacodynamic differences of AT1R blocker. One possibility is that CV lowers *E4BP4* mRNA expression (Fig. 5A), which produces a phase-dependent phase shift in ZG cells, as observed with Ang II-induced phase-dependent phase shifts in ZG region (Supplementary Fig. 3).

Collectively, in the present study, I have demonstrated that Ang II signaling can act as external/internal cues to modulate the molecular clock and circadian rhythm in adrenal ZG cells. Combined with previous data in our laboratory, my data provide a basis for understanding circadian properties of ZG cells as a component of body peripheral clocks. From a clinical perspective, optimizing the dosing schedules of CV not only minimizes potential side effects but may also improve ZG functions through restoring or enhancing clock function in the ZG. Therefore, my findings implicate a potential application of existing clinically used drugs to modulate the circadian clock in their specific target tissues and cells, opening up a new potential avenue in circadian medicine.

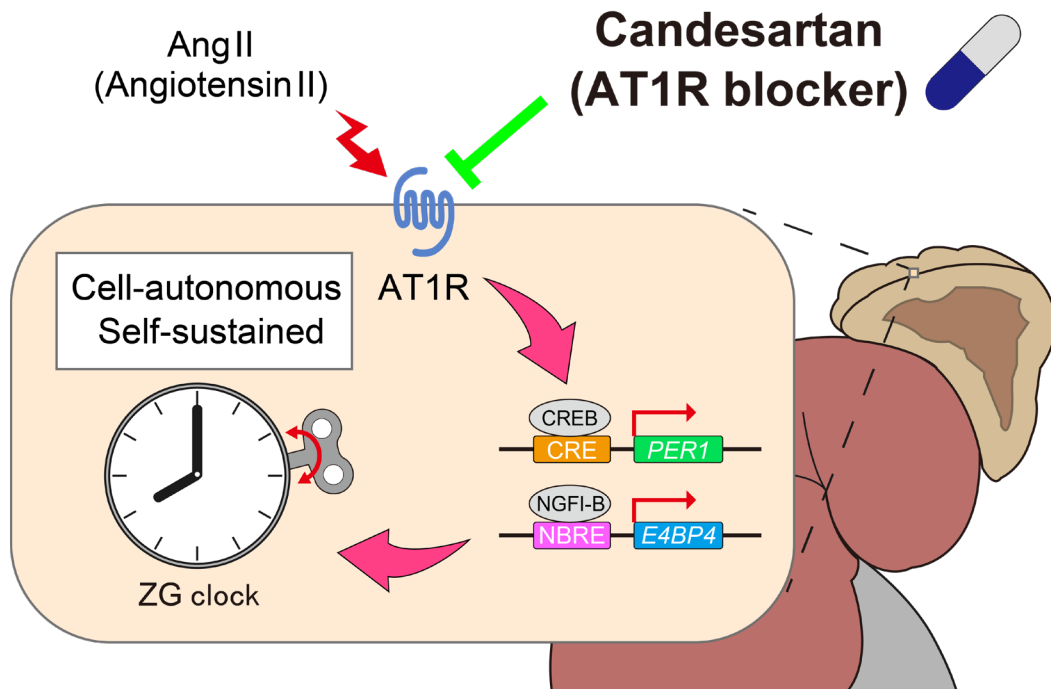


Fig. 6 | Schematics of the putative action of Ang II and CV on circadian oscillator in the adrenal ZG cells. A cell-autonomous and self-sustained clock is located in adrenal gland ZG cells. Ang II induces phase-dependent phase-shifts in the ZG clock through a mechanism involving Ang II type I receptor activation, which subsequently upregulates *PER1* and *E4BP4* mRNA expression. Candesartan, a widely prescribed AT1R blocker for hypertension treatment, may modulate the phase of the ZG clock during routine clinical use.

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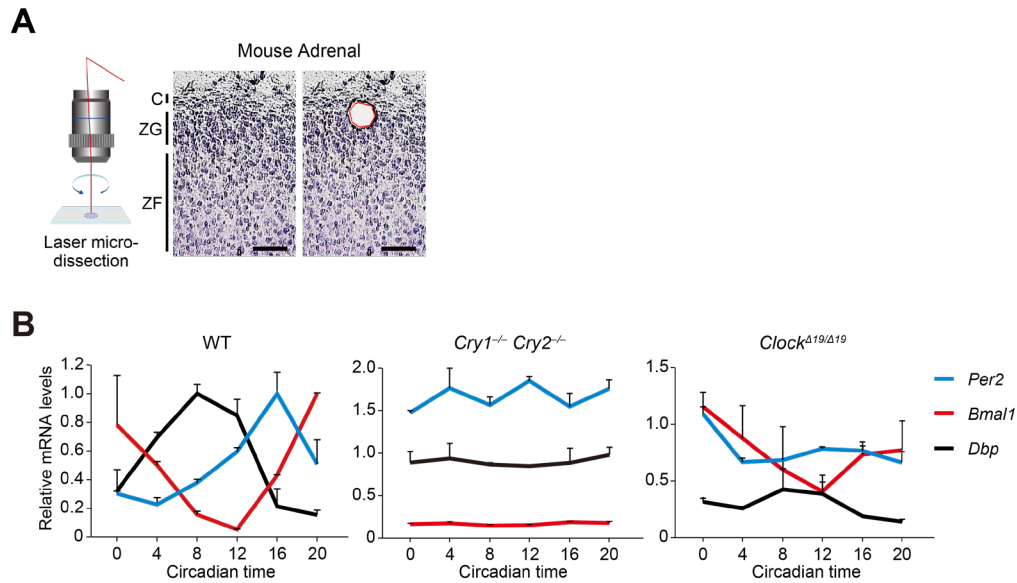
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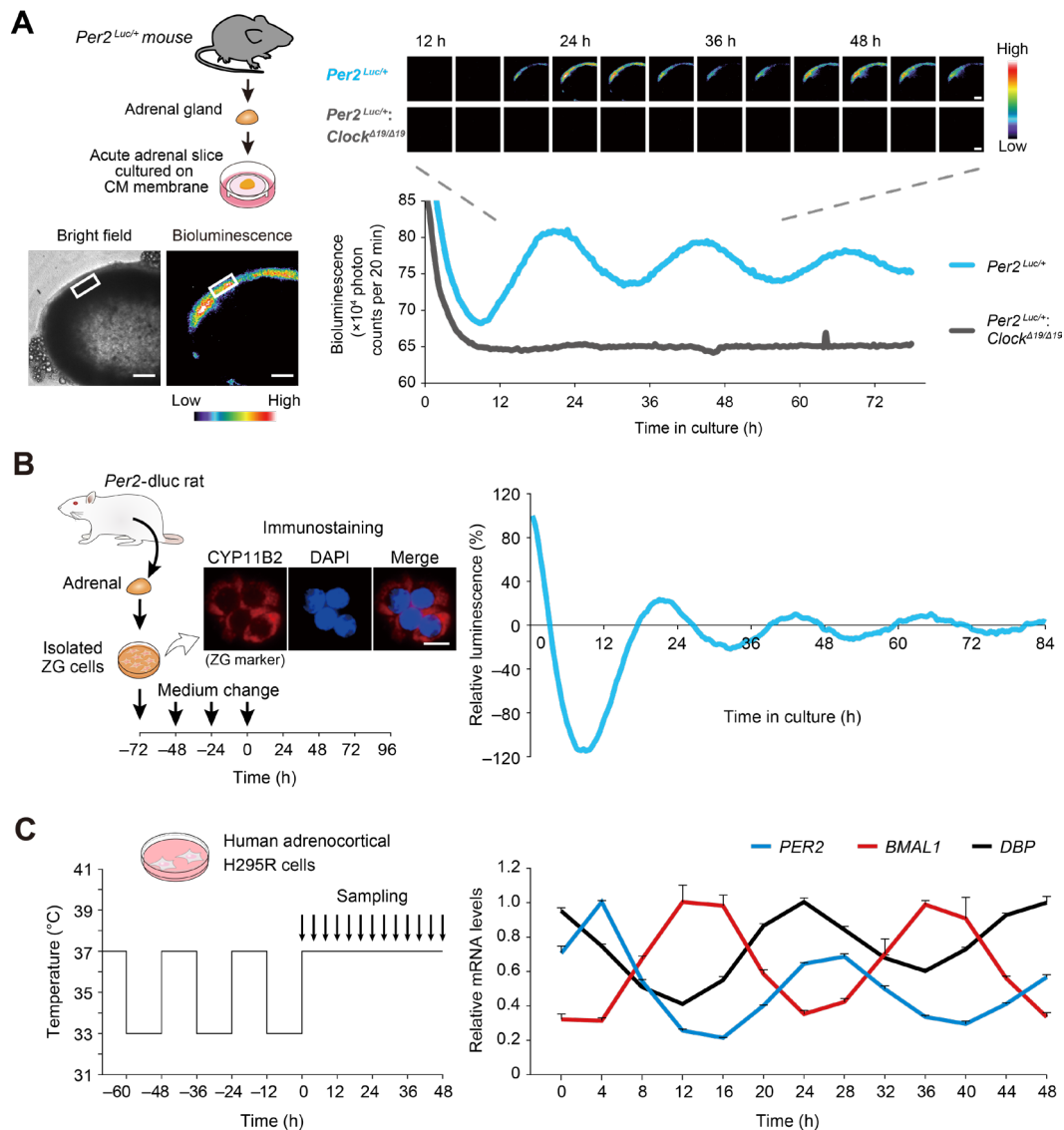
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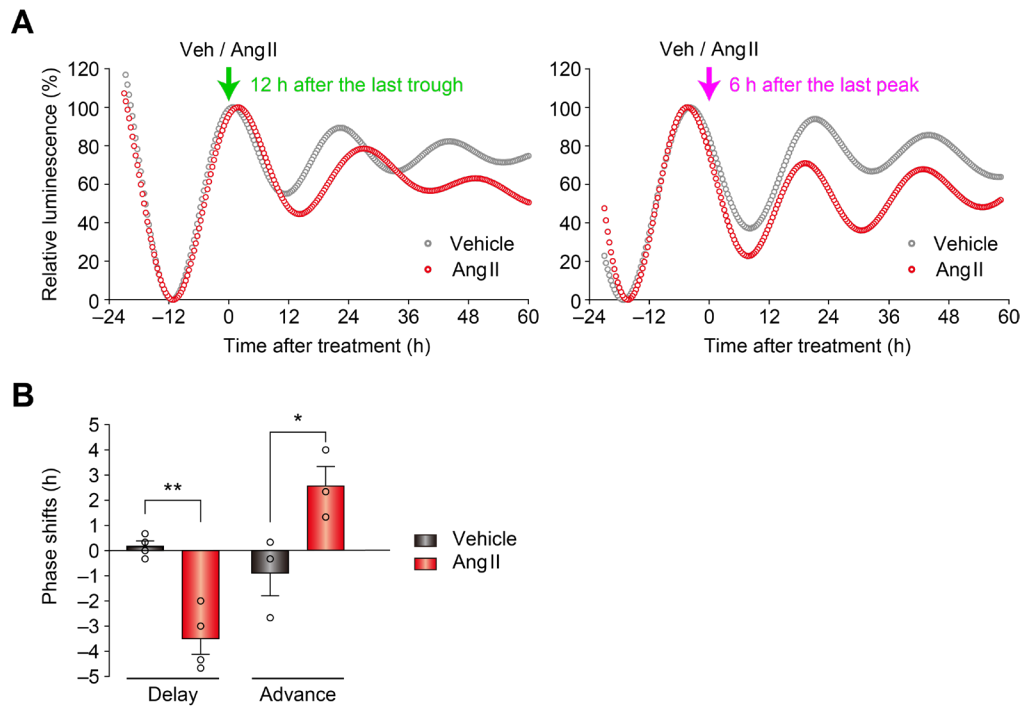
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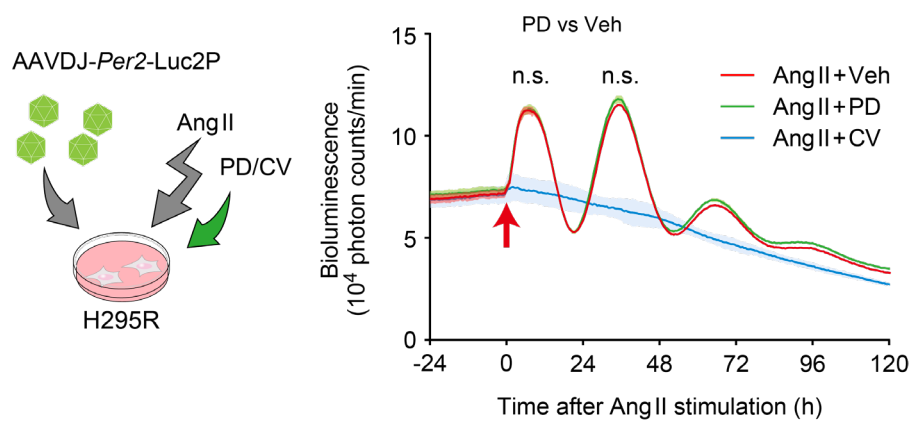
Supplementary Fig. 1 | Clock gene expression profiles in adrenal ZG cells from WT and circadian clock-deficient mice. (A) Representative adrenal cortex tissue section before and after laser micro-dissection. ZG cells are ovoid in shape and are arranged in globular clusters, lying directly beneath the capsular layer. C, capsule; ZF, zona fasciculata. Cresyl echt violet staining. Scale bars, 50 μ m. **(B)** Twenty-four-hour expression profiles of *Per2*, *Bmal1* and *Dbp* in wildtype, *Cry1^{-/-}Cry2^{-/-}*, and *Clock^{Δ19/Δ19}* adrenal ZG cells. Samples were collected at 4-h intervals for 24 h in DD using 2 mice per timepoint from each genotype. The peak values of each gene in WT adrenal ZG were set to 1. The levels of mRNA were determined by quantitative RT-PCR and expressed as the means $\pm$ variation normalized to those of the ribosomal phosphor-protein P0 (*Rplp0*)-encoding gene. (A) and (B) are reproduced from Ota *et al.* [97] with graphical modifications.



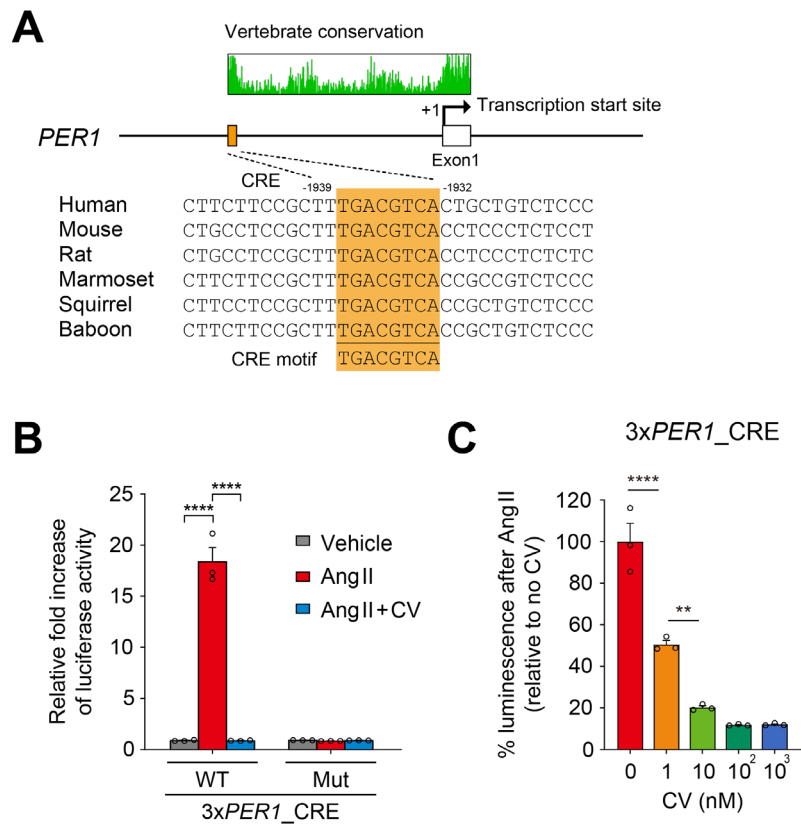
Supplementary Fig. 2 | Self-sustained circadian oscillations of in vitro cultured mouse, rat, and human ZG cells. (A) Time-lapse images of circadian *PER2::LUC* bioluminescence from *Per2^{Luc/+}* and *Per2^{Luc/+}; Clock^{Δ19/Δ19}* mouse adrenal slices. Intensity was traced from a region of the adrenal cortex outer layer containing ZG cells (white boxes) over 80 h. Bioluminescence intensity is represented in pseudo-colour scale. Scale bars, 200 μm . **(B)** *Per2-dluc* bio-luminescence from dissociated rat primary ZG cells. Isolated ZG cells were entrained by 24-h interval medium changes and then released into constant conditions with no medium change for 96 h. The data were detrended by 24-h moving average and shown relative to the value at the last medium change. Immunocytochemistry for CYP11B2 confirmed the isolation of ZG cells from rat adrenal glands. Scale bar, 10 μm . **(C)** Circadian oscillation of clock gene expression in the human adrenocortical H295R cells. Cells pre-synchronized to 37°C/33°C temperature cycles were harvested under constant 37°C temperature conditions. The data were normalized to *RPLP0*. The peak mRNA values of each gene were set to 1. $n = 3$ biological replicates per timepoint. Values are the means $\pm$ s.e.m. (A-C) are reproduced from Ota *et al.* [97] with graphical modifications.



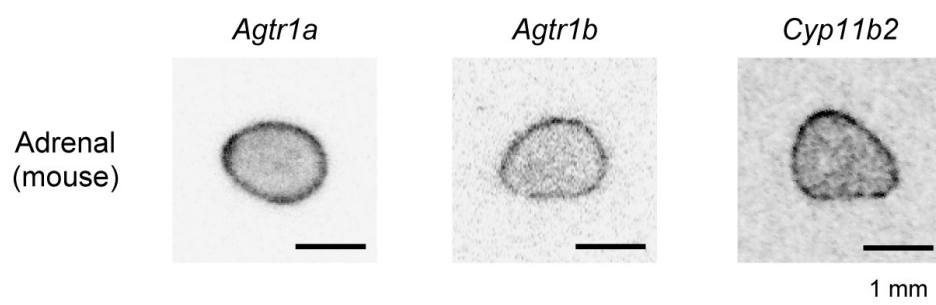
Supplementary Fig. 3 | Ang II elicits phase-dependent phase shifts of the adrenal ZG clock. (A) Phase shifts of PER2::LUC rhythm after Ang II treatment in adrenal slices. Arrows indicate the time of Ang II or vehicle administration. Luminescence of ZG was traced. The peak and trough values were adjusted to 100 and 0, respectively. **(B)** Quantification of the magnitude of phase shifts shown in (A). Phase delays and advances are plotted as negative and positive values, respectively. $n = 3-4$ slices per condition. Values are means $\pm$ s.e.m. $*P < 0.05$, $**P < 0.01$, unpaired two-sided Student's t test. (A) and (B) are reproduced from Ota *et al.* [97] after rigorous data reanalysis.



Supplementary Fig. 4 (related to Fig. 3) Absent effect of Ang II type 2 receptor blocker PD on Ang II-induced circadian bioluminescence in H295R cells. Experiments were performed as described in Fig. 2A using virally infected H295R cells. Traces indicate luminescence from cells harboring a luciferase reporter under the *Per2* promoter. PD, CV or vehicle (Veh) was administered together with Ang II at the time point indicated by the red arrow. The data for the treatment with Ang II and Veh (Ang II + Veh) are identical to those plotted in Fig. 2A. Data represent means $\pm$ s.d. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. n.s., not significant, PD vs. Veh for the first and second peak expression of luminescence.



Supplementary Fig. 5 (related to Fig. 4) | Cis-regulatory element involving Ang II-induced upregulation of *PER1* mRNA expression. **(A)** Sequence alignment of CRE located in the promoter of *PER1*. The sequences of CRE are compared among mammalian species along with the consensus CRE motif (5'-TGACGTCA-3'). Genomic positions, relative to the transcription start site (+1), are indicated along with the conservation scores obtained from the UCSC Genome Browser (<https://genome.ucsc.edu/>). **(B)** Relative reporter activities of CRE×3-luc and its mutant (Mut, TCACATAA). Cells received vehicle, Ang II, or Ang II plus CV (1 μM). *n* = 3 biological replicates. **(C)** Dose-dependent effects of CV on CRE reporter activity after Ang II stimulation. *n* = 3 biological replicates for each CV concentration. Statistics in (F) was one-way ANOVA followed by Tukey's multiple comparisons test; in (E), two-way ANOVA followed by Sidak's multiple comparison test. Values are means ± s.e.m.; ***P* < 0.01, *****P* < 0.0001.



Supplementary Fig. 6 (related to Fig. 5) | Autoradiographs showing expression of *Agtr1a*, *Agtr1b*, and *Cyp11b2* in the mouse adrenal section.

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