Supporting Information

Son *et al.* 10.1073/pnas.0806962106

SI Materials and Methods

Animal Care and Handling. Wild-type male C57BL/6J mice at 9–12 weeks of age were obtained from the Laboratory Animal Center at Seoul National University. Mice were kept in temperature-controlled (22 °C–23 °C) quarters under a 12-h light and 12-h dark (LD) photoperiod (lights on at 8:00 AM), with standard mouse chow and water available ad libitum. For dark–dark (DD) conditions, mice were kept in constant darkness for the indicated duration from the lights-off time. Home-cage activities and body temperatures were simultaneously monitored by using a VitalView data acquisition system (Mini Mitter) with implantable E-Mitters after entrainment for more than 10 days under LD conditions. All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University.

Plasmids. The expression vectors for clock genes were described previously (1, 2). Expression vectors for PERs and CRYs were kindly provided by H. Okamura (Kobe University Graduate School of Medicine, Kobe, Japan). Mouse MC2R (1.3 kb, −1236 to +98 from the transcription start site) and StAR (2.5 kb, −2527 to −1) promoter fragments were amplified from mouse genomic DNA by PCR. A Bmal1 cDNA fragment (+27 to +1618 of the coding sequence) was also prepared by PCR using mouse liver cDNA. Shorter and E3/4-mutated mouse StAR fragments were produced from the 2.5-kb StAR promoter fragment by PCR. In the E3/4-mutated StAR promoter, the distal E boxes (CACGTG) are mutated to CACTG (2). All of the PCR products were initially cloned into the pGEM-T Easy vector (Promega) and confirmed by chain termination sequencing. Luciferase reporter plasmids were prepared by inserting each fragment into the pGL3-basic vector (Promega) by using the XhoI/HindIII sites. Primer sequences used for cloning were as follows: MC2Rp up, 5′-CAT ATG TAG CTG GCC TTC TCT AAG-3′; MC2Rp dn, 5′-GCT AGC CTG AAG TAG GAT CTT TCT CG-3′; AS-Bmal1 up, 5′-CTC GAG CTC AAC CAT CAG CGA CTT CA-3′; AS-Bmal1 dn, 5′-TCT TCT TGC CTC CTG GAG AA-3′; StAR p2.5K up, 5′-GGT ACC CTC CTT CCG TGT AGG CTC TG-3′; StARp2.0K up, 5′-GGT ACC TCC TGA ACC AAA GGT GTG TG-3′; StARp1.5K up, 5′-GGT ACC ATG ACT AGT GAG CCC TGG CAC AAG AGT AG-3′; StARp1.0K up, 5′-GGT ACC CTG CAG GGA CTG ACA ACA GTC-3′; StARp dn, 5′-AAG CTT CTG AGT GCT GAG GTG CTC AAG-3′; StARp mutE34 up, 5′-GAA GGA GAG CCC ACT GTG TCT GGG GAA GCA CTG TAC C-3′; and StARp mutE34 dn, 5′-GGT ACA GTG CTT CCC CAG ACA CAG TGG GCT CTC CTT C-3′.

Cell Culture and Transfection. Materials for cell culture were obtained from Invitrogen. Immortalized adrenocortical Y1 cells were maintained in DMEM-F12K supplemented with 4 mM glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin/streptomycin, and 10% FBS in a humidified atmosphere containing 5% CO_2 at 37 °C. For serum shock, cells at 80–90% confluence were serum-starved for 24 h and incubated with 50% horse serum for 2 h, after which point they were returned to serum-free DMEM-F12K medium. The beginning of serum shock was defined as time 0. For transient transfection, the indicated plasmid DNAs were each introduced into 5×10^5 cells with the Microporation system (2 pulses of 25-msec duration at 1,400 V; Digital Bio Technology), except for the transfection experiments involving luciferase assays. Transfection efficiencies by electroporation were higher than 75%.

Generation of MC2R-AS-BMAL1 TG Mice. The inserted fragment containing the MC2R promoter, AS-BMAL1, and the bovine growth hormone poly(A) signal was cut out by NdeI/NcoI digestion and purified by agarose gel electrophoresis. The TG mice were generated by microinjection of the purified DNA into the pronuclei of fertilized eggs of C57BL/6J mice. TG animals were identified by PCR amplification of tail genomic DNA. PCR genotyping was carried out with the following 3 primers: MC2R promoter up for genotyping, 5′-ATA TGT TCC GGC CTT TCC TG-3′; AS-BMAL1 dn for genotyping, 5′-TTG GCG TAT CTA CCA CAG GA-3′; and endogenous MC2R dn for genotyping, 5′-TGG GAT AGG GAG TTT GTG GA-3′. To confirm adrenal gland-specific expression of the AS-BMAL1 transgene, competitive RT-PCR was performed on RNA samples from various tissues by using the following 3 primers: endogenous BMAL1 5′-UTR up, 5′-CAA GCA CCT TCC TTC CAA TG-3′; common BMAL1 dn, 5′-GAT TGC AGT CCA CAC CAC TG-3′; and transgene up 5′-AGT GGC ACC TTC CAG GGT CAA-3′.

Measurement of Steroids and Catecholamines. CS levels in adrenal lysates and plasma samples were assayed by using a commercial CS RIA kit (DPC) as described previously (3). Steroid production from Y1 cells was examined with the same commercial reagents by cross-reactivity. Plasma norepinephrine and epinephrine levels were measured with a commercial ELISA kit according to the manufacturer's instructions (Labor Diagnostika Nord).

RNA Isolation and RT-PCR. RNA analyses were performed as described previously with modifications (3). Mouse tissues except pancreas were rapidly removed, frozen in liquid nitrogen, and stored at −70 °C until use. Total RNA was isolated by the single-step acid guanidinium thiocyanate–phenol–chloroform method. The pancreas (which contains exceedingly high levels of RNase) was immediately homogenized in the guanidinium thiocyanate lysis buffer, and total RNA was isolated by using a commercial RNA extraction kit (Applied Biosystems). For Northern blot hybridization, a radioactive cDNA probe for each gene was generated by random priming in the presence of [α-^{32}P]dCTP by using cDNA fragments for StAR, p450scc, 3βHSD, SF-1, Dax-1, or 18S rRNA. Hybridization was performed overnight at 42 °C. After washing off unbound probe, the membranes were exposed to X-ray film at −70 °C for 3 days. For RT-PCR, 500 ng of each RNA sample was reverse-transcribed with MMLV reverse transcriptase (Promega). Then, aliquots of the cDNA were subjected to quantitative real-time PCR in the presence of SYBR Green I (Sigma). Gene expression levels were normalized with TATA box-binding protein (TBP). Primer sequences used for real-time RT-PCR were as follows: StAR up, 5′-TTG GGC ATA CTC AAC AAC CA-3′; StAR dn, 5′-GAA ACA CCT TGC CCA CAT CT-3′; Per1 up, 5′-GTG TCG TGA TTA AAT TAG TCA G-3′; Per1 dn, 5′-ACC ACT CAT GTC TGG GCC-3′; Per2 up: 5′-ATG CTC GCC ATC CAC AAG A-3′; Per2 dn: 5′-GCG GAA TCG AAT GGG AGA AT-3′; TBP up: 5′-GGG AGA ATC ATG GAC CAG AA-3′; and TBP dn: 5′-CCG TAA GGC ATC ATT GGA CT-3′.

In Situ Hybridization. Mouse brain samples were immediately frozen in prechilled isopentane. Frozen sections (12 μm thick)

were cut coronally and thaw-mounted onto gelatin-coated slides. The sections were fixed in 4% formaldehyde, dehydrated in increasing concentrations of ethanol, and air-dried. Antisense cRNA probes were prepared by transcription with RNA polymerase using a Riboprobe system (Promega). The sections were hybridized overnight at 52 °C with 1.2×10^6 cpm of labeled RNA probe per slide. They were then washed in 4× SSC, treated with RNase A (20 μg/mL; Boehringer–Mannheim), and washed several times. After drying, the slides were autoradiographed for 7 days using β-max Hyperfilm (Amersham Biosciences).

Western Blot Analysis. Anti-StAR (Abcam) and actin (Sigma) antibodies were obtained commercially. Anti-BMAL1 antibody was raised by immunizing rabbits and was affinity-purified as described previously (4). Whole-cell extracts were resolved on SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore) in a Bio-Rad TransBlot electrophoresis apparatus using Towbin's buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol). The blots were blocked in Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris, pH 7.6, and 2 mM $MgCl_2$) containing 0.3% Tween 20 and 3% BSA and were incubated with primary antibody at room temperature for 1 h. They were then washed 4 times with TBS/0.3% Tween-20. Antibody binding was subsequently detected by incubation with secondary antibodies linked to horseradish peroxidase (Jackson ImmunoResearch). The blots were washed 4 times as described, and immunoreactive bands were visualized by exposure to X-ray film for 0.5 or 2 min after application of Amersham ECL reagents (Amersham Biosciences). Optical densities of immunoreactive bands were quantified by using National Institutes of Health ImageJ software (downloaded from http://rsb.info.nih.gov/ij/), and then the relative amounts of target proteins were deduced by comparison with the optical band densities from serially diluted reference extracts.

Luciferase Assay. Each of the luciferase reporter and expression vectors for the clock genes were transfected together with 100 ng of a thymidine kinase promoter-driven *Renilla* luciferase (pRL-TK) construct by using Lipofectamine PLUS reagent (Invitrogen). Twenty nanograms of reporter DNA and 200 ng of each clock gene were used. The total amount of DNA used was held constant by adding pcDNA3.1 plasmid. After 48 h of transfection, cell extracts were prepared by incubation in 0.3 mL of reporter lysis buffer (Promega) for 15 min at room temperature. Luciferase activities were measured with a commercial dual luciferase assay kit (Promega).

ChIP Assay. ChIP assays were performed by using a commercial ChIP assay kit (Upstate Biotechnology). Sheared and precleared chromatin from naïve Y1 cells was immunoprecipitated for 2 h at 4 °C by agitating with 2 μg of anti-BMAL1 or anti-acetylated histone H3 (AcH3) antibody (Upstate Biotechnology). As a negative control, the supernatant was incubated with 5 μL of preimmune normal rabbit serum (NRS). Immune complexes were collected by incubation with Protein-A Sepharose beads. The primer sets used were: StAR E3/4 up, 5′-TCC TGA ACC AAA GGT GTG TG-3′; StAR E3/4 dn, 5′-GAA ACT CCC TCA CTG GTG TTC-3′; StAR E2 up, 5′-CTG AGA TAC CTA GCA AGT TC-3′; StAR E2 dn, 5′-GCA GTT AAG TCC ACT GAC TG-3′; StAR E1 up, 5′-AGT AGC CTC AAA GGT AC-3′; StAR E1 dn, 5′-GAC TGT TGT CAG TCC CTG CAG-3′; DBP E box up, 5′-CGC GCA AAG CCA TGT GCT TCC CCC T-3′; and DBP E box dn, 5′-AGG GGG AAG CAC ATG GCT TTG CGC G-3′.

1. Yagita K, *et al.* (2002) Nucleocytoplasmic shuttling and mCRY-dependent inhibition of ubiquitylation of the mPER2 clock protein. *EMBO J* 21:1301–1314.
2. Jung H, *et al.* (2003) Involvement of CLOCK:BMAL1 heterodimer in serum-responsive mPer1 induction. *Neuroreport* 14:15–19.
3. Chung S, *et al.* (2005) Differential adaptive responses to chronic stress of maternally stressed male mice offspring. *Endocrinology* 146:3202–3210.
4. Kwon I, *et al.* (2006) BMAL1 shuttling controls transactivation and degradation of the CLOCK/BMAL1 heterodimer. *Mol Cell Biol* 26:7318–7330.

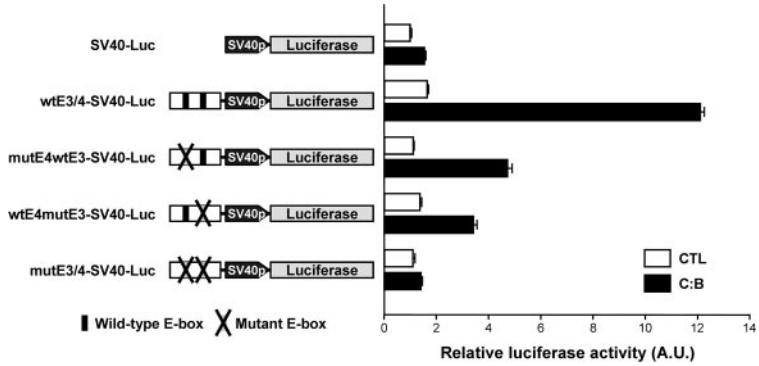

Fig. S1. Effect of mutation(s) on the distal E boxes. A StAR promoter fragment (−2175 to −1885, 190 nucleotides) including the 2 distal E boxes (denoted E3 and E4) was fused to the SV40 minimal promoter–luciferase reporter construct (SV40-Luc) to generate wtE34-SV40-Luc. The constructs with mutated E box (CACGTG to CAGTG) are denoted mutE4wtE3-SV40-Luc, wtE4mutE3-SV40-Luc, and mutE34-SV40-Luc, respectively. Twenty nanograms of each luciferase reporter plasmid was cotransfected with a 1:1 mixture of CLOCK and BMAL1-expressing (200 ng each; C:B) plasmids or 400 ng of backbone plasmid (pcDNA3.1; CTL). pRL-TK (100 ng) was used for normalization of transfection efficiencies. After 48 h of transfection, cells were lysed, and luciferase assays were performed. Luciferase activities are shown as mean ± SE of arbitrary units (A.U.), where the mean luciferase activities from backbone plasmid and SV40-Luc-transfected cells are defined as 1. Elimination of either E3 or E4 partially reduced the C:B-evoked enhancing effect of the StAR promoter fragment on SV40 minimal promoter. These results indicate that both E3 and E4 are functional and work additively.

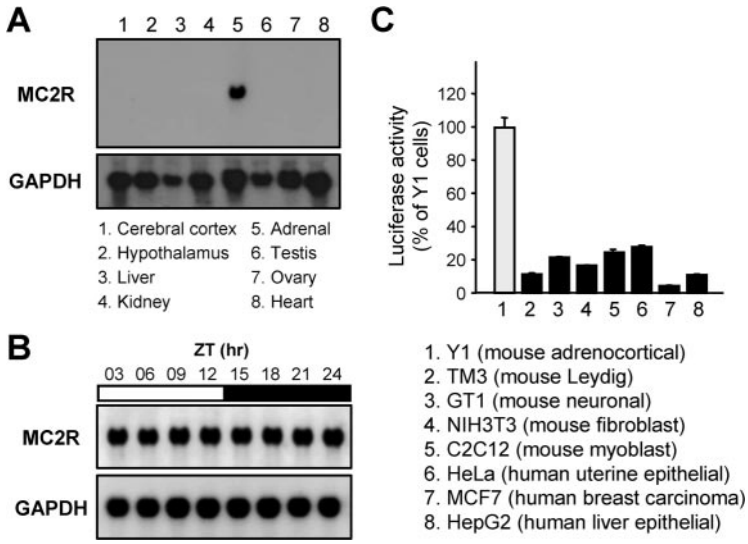

Fig. S2. The MC2R promoter used for the adrenal gland-specific expression of a transgene. (*A*) Northern blot analysis of various mouse tissues with a radiolabeled probe against the ACTH receptor (MC2R). MC2R mRNA was detected exclusively in the adrenal gland. (*B*) MC2R mRNA profiles in adrenal glands at different times. MC2R gene expression was constant throughout the day. (*C*) The promoter region of the MC2R gene (−1236 to +98) was amplified by PCR and fused to a firefly luciferase reporter to generate the pMC2R-Luc reporter construct. Twenty nanograms of pMC2R-Luc was cotransfected with pRL-TK into a variety of mouse and human cell lines as indicated. After 48 h, the cells were lysed and dual luciferase assays were performed. Luciferase activities from MC2Rp-Luc were normalized with those from pRL-TK. Relative luciferase activities are presented as mean ± SE % of the activity of Y1 ($n = 3$).

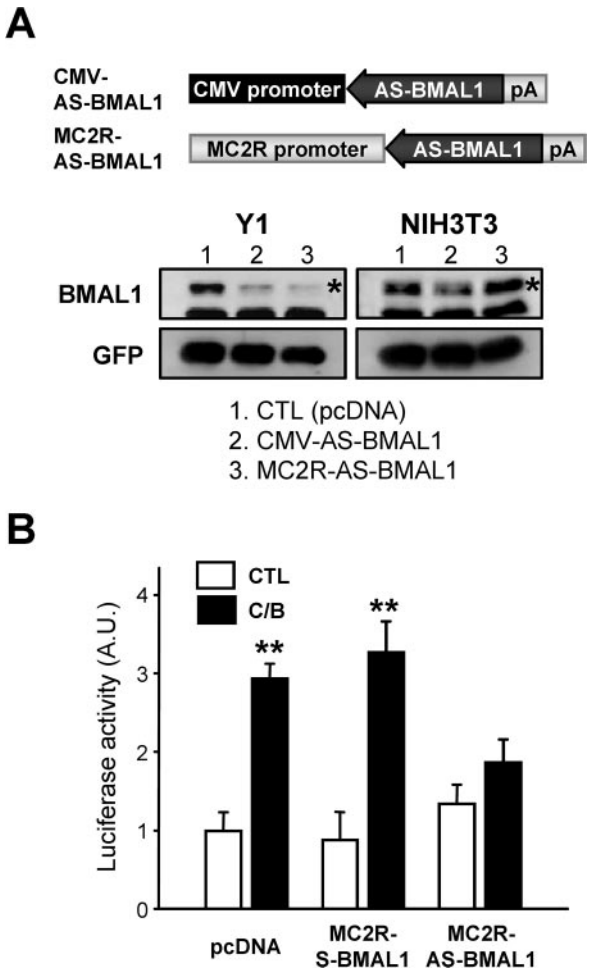

Fig. S3. Efficient knockdown of BMAL1 with AS-BMAL1 constructs. (*A*) Y1 and NIH 3T3 cells were transfected with 20 ng each of control (CTL), CMV promoter-driven antisense BMAL1 (+27 to +1618; pCMV-AS-BMAL1), or pMC2R-AS-BMAL1 in combination with 300 ng of pEGFP-N1 plasmid (Stratagene) by Microporation. After 24 h, immunoblot analyses were carried out with anti-BMAL1 antibody. Cotransfected GFP was used as an internal control for amounts of protein and transfection efficiencies. Asterisks indicate the endogenous BMAL1 proteins. (*B*) Effect of pMC2R-AS-BMAL1 and pMC2R-S-BMAL1 on CLOCK:BMAL1-evoked induction of 6.8-kb mouse *Per1* promoter activity. Twenty nanograms of *mPer1* promoter-driven luciferase reporter plasmid was cotransfected with 400 ng of control (CTL; pcDNA3.1) or a 1:1 mixture of CLOCK and BMAL1-expressing constructs (C:B). A total of 400 ng of control (pcDNA), pMC2R-AS-BMAL1, or pMC2R-S-BMAL1 plasmid was also added to each set of cells. After 48 h, *Per1* promoter-driven luciferase activities were measured and normalized with those of cotransfected pRL-TK. Data are presented as mean ± SE of arbitrary units, where the mean value of CTL with pcDNA is defined as 1 ($n = 8$ for each set). $*P < 0.01$ between CTL and C:B in the same set.

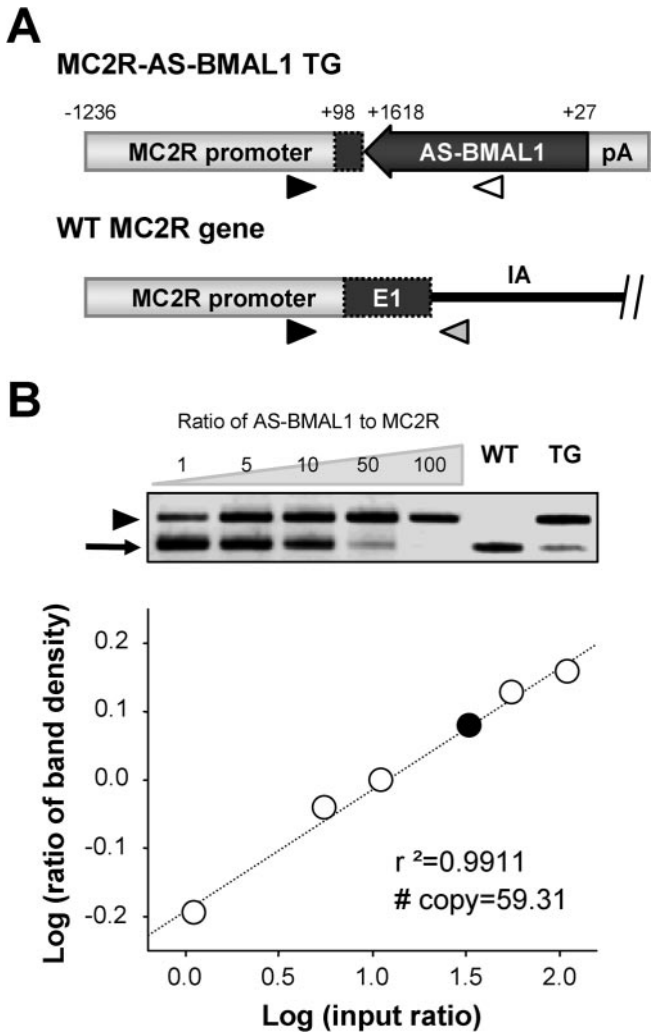

Fig. S4. Determination of the transgene copy number. (*A*) Schematic diagrams of the MC2R-AS-BMAL1 transgene and endogenous MC2R gene and their primer binding sites. (*B*) Competitive PCR using 3 primers was carried out as described previously [Seong JY, *et al.* (2001) First intron excision of GnRH pre-mRNA during postnatal development of normal mice and adult hypogonadal mice. *Endocrinology* 142:4454–4461]. Titration and constructing standard curve of competitive PCR for the quantification of AS-BMAL1 and endogenous MC2R were performed in the presence of 1 pg of purified PCR product from WT MC2R gene and serial dilutions of those from AS-BMAL1 transgene (1–100 pg as indicated). By linear regression, the average transgene copy number in a pooled genomic DNA from 3–6 heterozygote animals was calculated. The mean copy number from 3 independent performances was 58.66 ± 0.39.

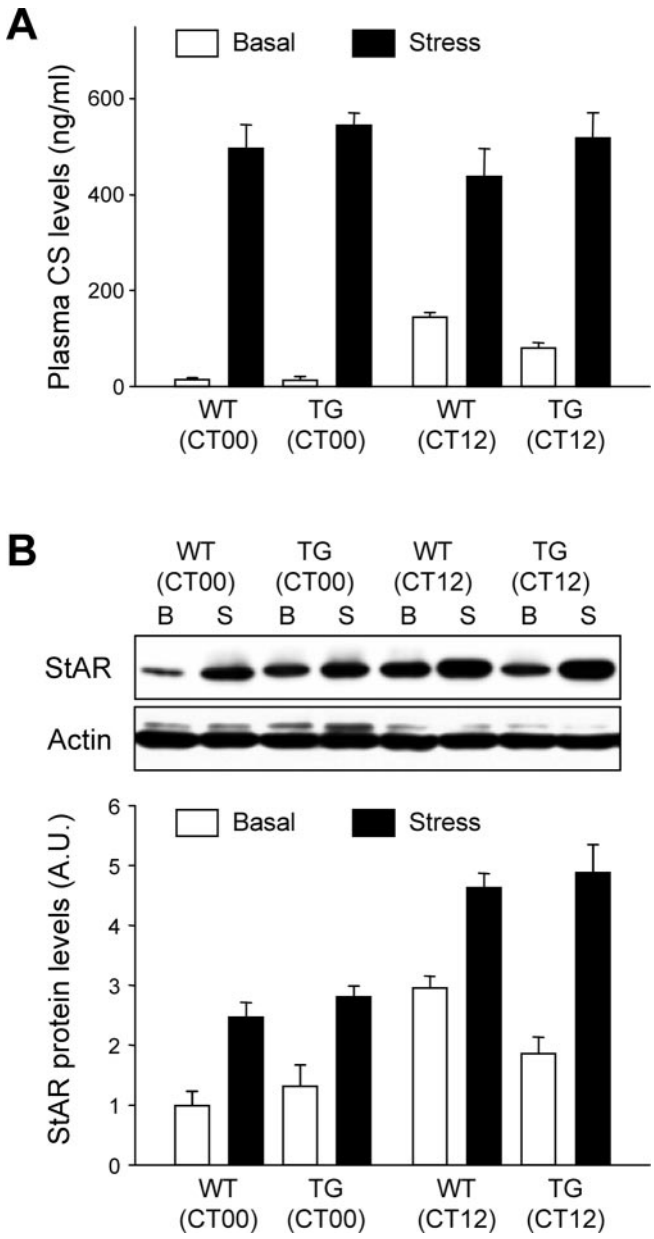

Fig. S5. Stress-induced plasma CS levels and StAR protein expression. WT and TG mice kept under DD conditions for 6 days were undisturbed (Basal) or immobilized for 30 min (Stress) at CT00 or CT12. (*A*) Plasma CS levels were measured by RIA. Data are expressed as mean ± SE ($n = 6–9$ for each group). (*B*) (*Upper*) Adrenal StAR protein levels were determined by Western blotting. (*Lower*) Relative StAR protein levels are presented as mean ± SE of A.U. ($n = 4$ for each group). Immobilization stress produced significant increases in both plasma CS levels and StAR protein expression. Although the extent of fold-inductions in CS and StAR levels was not exactly correlated, stress-induced CS and StAR increments were not significantly different between WT and TG mice.

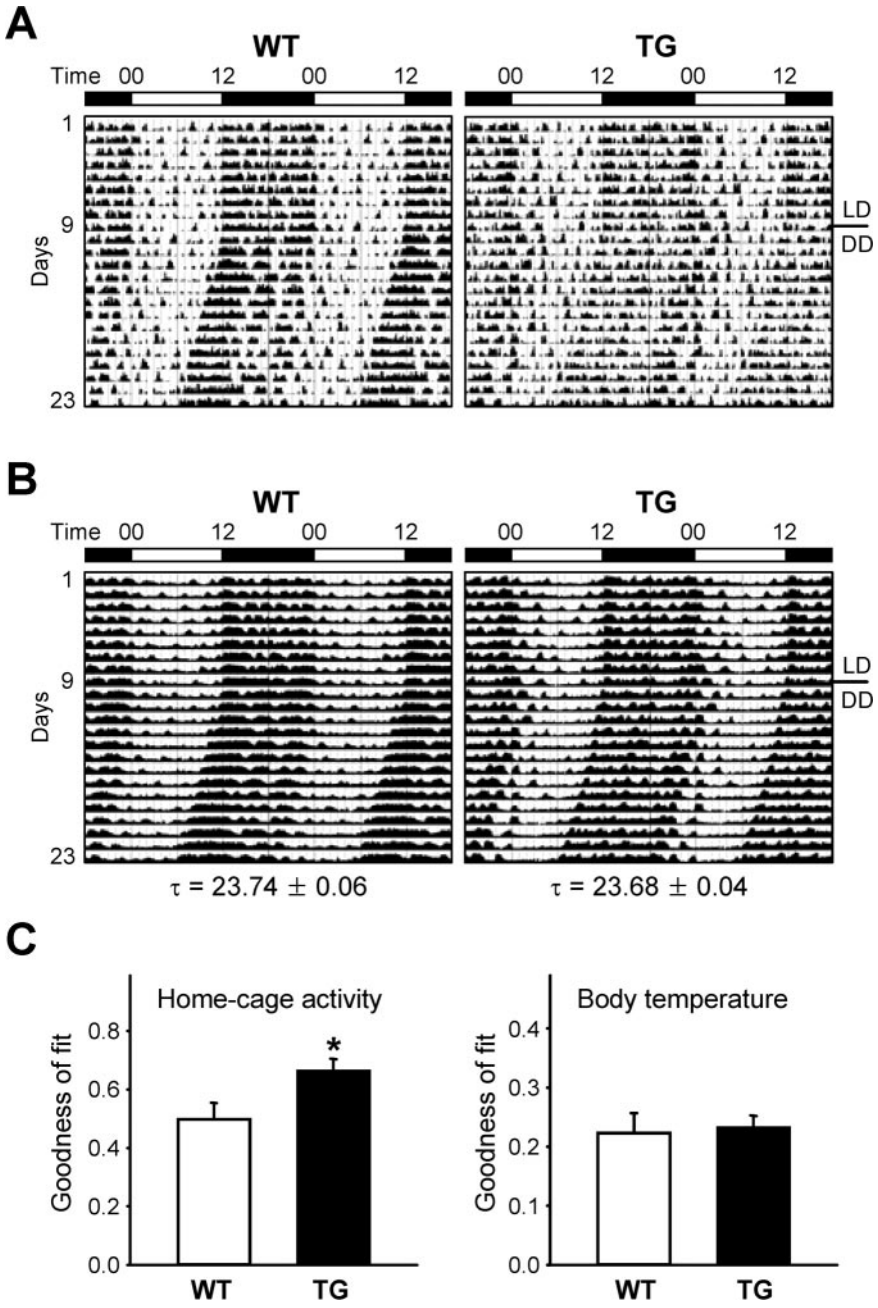

Fig. S6. Impaired behavioral rhythm, but normal circadian variation in body temperature. (*A*) Representative double-plotted actograms for the home-cage activities of WT and TG mice. (*B*) Changes in body temperature measured s.c. are double-plotted by monitoring day. Temperatures are indicated from 34 °C to 38 °C. There was no significant difference between WT and TG mice in either the period or amplitude of the rhythmicity. (*C*) Regression analyses with 24-h period into cosine functions were performed on the daily rhythms of home-cage activities (*Left*) and body temperature (*Right*) under DD conditions. Data are expressed as means ± SE of the goodness of fit for the regression curve. Both genotypes elicited similar goodness of fit values for changes in body temperature. By contrast, TG mice showed significantly increased values for goodness of fit in their home-cage activity, indicating that their rhythms were weakened compared with those of their WT littermates.

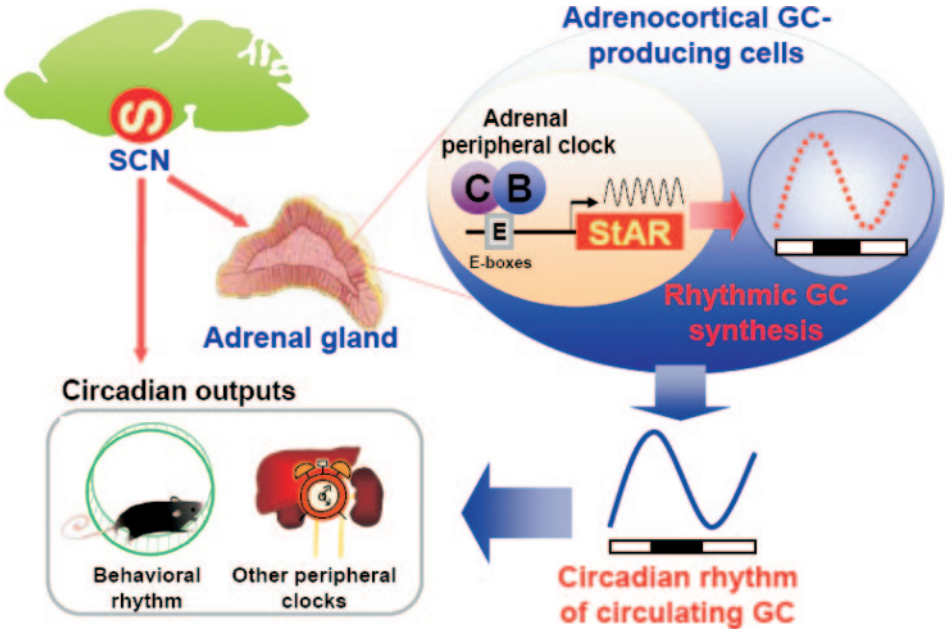

Fig. S7. Role of the adrenal peripheral oscillator in circadian GC production and its physiological relevance. Cyclic expression of StAR is directly controlled by CLOCK:BMAL1 heterodimer, and the resulting daily oscillation in steroidogenesis contributes to the generation of a robust GC rhythm. Then, GC can play a pivotal role in synchronizing and maintaining the mammalian circadian timing system by influencing behavioral rhythm and the periodic expression of clock genes in other peripheral organs.

Table S1. Basal and stress-induced plasma norepinephrine and epinephrine levels

		Norepinephrine, ng/ml		Epinephrine, ng/ml	
Genotype	CT, h	Basal (*n*)	Stress (*n*)	Basal (*n*)	Stress (*n*)
WT	00	4.79 ± 1.20 (18)	12.59 ± 3.57 (5)*	0.64 ± 0.15 (18)	2.56 ± 0.44 (5)**
	06	3.66 ± 0.87 (16)	ND	0.62 ± 0.15 (16)	ND
	12	5.05 ± 1.14 (18)	10.52 ± 2.92 (6)*	0.87 ± 0.32 (18)	2.50 ± 0.78 (6)*
	18	5.69 ± 1.85 (16)	ND	0.57 ± 0.14 (16)	ND
TG	00	5.24 ± 1.07 (17)	10.94 ± 2.63 (5)*	0.87 ± 0.24 (17)	2.22 ± 0.94 (5)**
	06	5.23 ± 1.15 (15)	ND	0.71 ± 0.21 (15)	ND
	12	3.90 ± 0.71 (17)	10.11 ± 1.18 (6)*	0.61 ± 0.17 (17)	1.77 ± 0.16 (6)*
	18	4.10 ± 0.84 (15)	ND	0.46 ± 0.11 (15)	ND

WT and TG mice kept under DD conditions for 6 days were undisturbed (Basal) or immobilized for 30 min (Stress) at CT00 or CT12. Plasma norepinephrine (NE) and epinephrine (E) levels were measured with a commercial ELISA kit according to the manufacturer's instructions (Labor Diagnostika Nord). Plasma samples for basal catecholamines were prepared from 4 independent batches of animals and for stress-evoked levels from 2 independent batches. Data are expressed as mean ± SE. *, $P < 0.05$, and **, $P < 0.01$ between basal and stress-induced levels. Two-way ANOVA revealed that there were no significant differences by genotypes and sampling time in spite of slight, but not significant, daily variations. No apparent daily rhythm might be produced by their modest amplitudes, but larger variations among individual animals compared with those seen in CS. Considering a drastic impairment of circulating NE and E levels in global BMAL1$^{-/-}$ mice [Curtis AM, *et al.* (2007) Circadian variation of blood pressure and the vascular response to asynchronous stress. *Proc Natl Acad Sci USA* 104:3450–3455], these results strongly suggest that our strategy using MC2R promoter did not significantly affect adrenal medulla, or an extra-adrenal clock including the central clock might be a primary determinant of the basal NE and E levels.