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Chronic mild stress alters circadian expressions of molecular clock genes in the liver

Kei Takahashi,[1,2] Tetsuya Yamada,[1] Sohei Tsukita,[1,2] Keizo Kaneko,[2] Yuta Shirai,[1,2] Yuichiro Munakata,[1,2] Yasushi Ishigaki,[2] Junta Imai,[2] Kenji Uno,[2] Yutaka Hasegawa,[2] Shojiro Sawada,[2] Yoshitomo Oka,[2] and Hideki Katagiri[1]

[1]*Department of Metabolic Diseases, Center for Metabolic Diseases, Tohoku University Graduate School of Medicine, Sendai, Japan; and* [2]*Division of Molecular Metabolism and Diabetes, Tohoku University Graduate School of Medicine, Sendai, Japan*

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Takahashi K, Yamada T, Tsukita S, Kaneko K, Shirai Y, Munakata Y, Ishigaki Y, Imai J, Uno K, Hasegawa Y, Sawada S, Oka Y, Katagiri H. Chronic mild stress alters circadian expressions of molecular clock genes in the liver. *Am J Physiol Endocrinol Metab* 304: E301–E309, 2013. First published December 4, 2012; doi:10.1152/ajpendo.00388.2012.—Chronic stress is well known to affect metabolic regulation. However, molecular mechanisms interconnecting stress response systems and metabolic regulations have yet to be elucidated. Various physiological processes, including glucose/lipid metabolism, are regulated by the circadian clock, and core clock gene dysregulation reportedly leads to metabolic disorders. Glucocorticoids, acting as end-effectors of the hypothalamus-pituitary-adrenal (HPA) axis, entrain the circadian rhythms of peripheral organs, including the liver, by phase-shifting core clock gene expressions. Therefore, we examined whether chronic stress affects circadian expressions of core clock genes and metabolism-related genes in the liver using the chronic mild stress (CMS) procedure. In BALB/c mice, CMS elevated and phase-shifted serum corticosterone levels, indicating overactivation of the HPA axis. The rhythmic expressions of core clock genes, e.g., *Clock*, *Npas2*, *Bmal1*, *Per1*, and *Cry1,* were altered in the liver while being completely preserved in the hypothalamic suprachiasmatic nuculeus (SCN), suggesting that the SCN is not involved in alterations in hepatic core clock gene expressions. In addition, circadian patterns of glucose and lipid metabolism-related genes, e.g., *peroxisome proliferator activated receptor* (*Ppar*) α, *Pparγ-1*, *Pparγ-coactivator-1α*, and *phosphoenolepyruvate carboxykinase*, were also disturbed by CMS. In contrast, in C57BL/6 mice, the same CMS procedure altered neither serum corticosterone levels nor rhythmic expressions of hepatic core clock genes and metabolism-related genes. Thus, chronic stress can interfere with the circadian expressions of both core clock genes and metabolism-related genes in the liver possibly involving HPA axis overactivation. This mechanism might contribute to metabolic disorders in stressful modern societies.

stress; liver clock; metabolic disorders; hypothalamus-pituitary-adrenal axis

VARIOUS BEHAVIORAL AND PHYSIOLOGICAL processes, including feeding behavior and energy metabolism, exhibit circadian (i.e., 24-h) rhythmicity, which may play a role in maintaining functional homeostasis. These rhythms are regulated by the circadian clock system, which is composed of transcriptional/translational feedback loops. Although the mammalian master pacemaker is located in the hypothalamic suprachiasmatic nucleus (SCN), the core clock machinery has been identified in almost all peripheral tissues, including the liver (41). In brief, each cell contains a set of core clock genes, such as *Clock*, *Bmal1*, *Cry 1–2*, *Per 1–3*, and nuclear receptors (*Rev-erbα*, *ROR*). The CLOCK/BMAL1 heterodimer regulates the production of proteins such as PERs and CRYs, which in turn regulate the production of BMAL1 (44). Through these feedback loops, the expressions of core clock genes generate endogenous rhythms of numerous protein expressions, leading to rhythmic functioning of cells and tissues over an ~24-h period (12). The molecular clock has been demonstrated to modulate energy metabolism by controlling the expressions and activities of numerous enzymes, transport systems, and nuclear receptors involved in lipid and carbohydrate metabolism (41, 47, 55).

The prevalence of the metabolic syndrome, which represents a spectrum of metabolic and cardiovascular disorders, continues to increase at an alarming rate in modern societies. Responses to an unbalanced diet and lack of physical exercise increase the risk of this pathological condition. In addition, recent evidence suggests that metabolic disorders are associated with the settings of the circadian clock system (6, 26), and moreover that alterations in these settings are among the possible causes of metabolic disorders. For instance, homozygous *Clock* mutant mice become obese and develop metabolic syndrome (50). Disruption of *Rev-erbα* in mice leads to dysregulation of several genes involved in lipid metabolism and alters serum lipid profiles (30). A study using tissue-specific ablation of *Bmal1* demonstrated that clocks in the liver do indeed contribute to glucose homeostasis (29).

Stress is defined as any situation capable of perturbing physiological or psychological homeostasis (38). Health disorders that are associated with dysfunction of the stress system are now major problems in western societies (9). The stress response system consists of the hypothalamus-pituitary-adrenal (HPA) axis and its end-effectors, glucocorticoid receptors. Organisms have developed behavioral and physiological adaptations to the strong influences of day/night cycles, as well as to unforeseen, random stress stimuli. In addition, stress responses and the circadian clock communicate with one another at different signaling levels, resulting in interrelated regulatory networks, and dysregulation of either system can lead to the development of metabolic disorders (10, 35). Indeed, glucocorticoids, which are well known to affect metabolic conditions, entrain the circadian rhythm by phase-shifting the expressions of several core clock genes in peripheral organs, including the liver, kidneys, and heart (5). Therefore, we hypothesized that chronic stress affects the circadian rhythm of core clock gene expressions in the liver via the HPA axis, followed by alterations in metabolism-related genes. The

Address for reprint requests and other correspondence: T. Yamada, Dept. of Metabolic Diseases, Center for Metabolic Diseases, Tohoku Univ. Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan (e-mail: yamatetsu-tky@umin.ac.jp).

chronic mild stress (CMS) procedure is widely used for analyzing stress effects in experimental animal models (34, 38, 39, 53). To test our hypothesis, we examined the influences of CMS on circadian clock gene expressions in the liver.

MATERIALS AND METHODS

Animals. Eight-week-old male C57BL/6 mice and BALB/c mice (CLEA Japan, Tokyo, Japan) were obtained and maintained under specific pathogen-free conditions. Mice were maintained under controlled temperature and humidity on a 12:12-h light (0900–2100)-dark (2100–0900) cycle. They were housed individually and given a standard laboratory diet (65% carbohydrate, 4% fat, 24% protein) and water ad libitum unless otherwise noted. At 9 wk of age, they were divided into two groups, one continuously housed as stated above (control) and the other subjected to 1 wk of unpredictable CMS. At 10 wk of age, 24 mice of each strain were killed by cervical dislocation at 6-h intervals over 24 h (2, 4, 28, 46) to obtain liver and brain samples at zeitgeber times (ZTs) 3, 9, 15, and 21 in which ZT 0 is defined as lights on and ZT 12 as lights off (n = 6/group for each observation point). All animal studies were conducted in accordance with the institutional guidelines for animal experiments and were approved by Tohoku University.

CMS procedure. The stress protocol was slightly modified from those used in previous reports (34, 38). Briefly, there was one 16-h period of water deprivation; two periods of continuous overnight illumination; two periods (7 and 17 h) of 45° cage tilt; one 17-h period in a cage without bedding chips; one period (8 h) of food deprivation; and one 17-h period of paired housing. All individual stressors used had been classified as "mild" according to the Animals (Scientific Procedures) Act of 1986 (United Kingdom legislation). Animals were left undisturbed in their home cages for 24 h before death.

Measurement of food intake. Food intake was measured during the day after the last day of the CMS procedure, based on division into light (ZT 0 through ZT 12) and dark (ZT 12 through ZT 0) phases.

Blood analysis. Blood glucose levels were assayed with Antsense II (Horiba Industry, Kyoto, Japan) (51). Serum insulin and leptin levels were determined with enzyme-linked immunosorbent assay (ELISA) kits (Morinaga Institute of Biological Science, Yokohama, Japan) (51). Plasma corticosterone levels were assessed using an ELISA kit (ASSAYPRO, St. Charles, MO).

Laser microdissection. All animals were killed by cervical dislocation at the indicated ZTs. The brains were immediately frozen in isopentane on dry ice and stored at −80°C until RNA purification. Coronal cryostat sections (25 μm) through the bilateral SCN of the hypothalamus were placed on PEN-coated slides (Leica Microsystems). Laser microdissections were carried out on a Leica AS LMD (Leica Microsystems) (26). Immediately after microdissection, total RNA was purified, as described below.

RNA purification and quantitative real-time PCR. Total RNA from the liver was purified using an RNeasy minikit (QIAGEN, Valencia, CA) (51). Total RNA from laser microdissected SCN specimens was purified using an RNeasy microkit (QIAGEN) (26). cDNA synthesized from total RNA was evaluated using a real-time PCR quantitative system (Light Cycler Quick System 350S; Roche Diagnostics, Mannheim, Germany) as previously reported (51). Expression levels were normalized against the levels of *β-actin* (for liver) or *gapdh* (for SCN). The sequences of forward and reverse primers were as follows: *Clock*, forward, 5′-AGATCAGTTCAATGTCCTCA-3′; *Clock*, reverse, 5′-TGTCGAATCTCACTAGCATC-3′; *Bmal1*, forward, 5′-ATGAACCCGTGGACCAAG-3′; *Bmal1*, reverse, 5′-CCTGGAATGCCTGGAACA-3′; *Npas2*, forward, 5′-CAGCAGCCACCACCCTATT-3′; *Npas2*, reverse, 5′-TGCGGAGGTGTAGACTGTGT-3′; *Per1*, forward, 5′-GTACTTTGGCAGCATCGACTC-3′; *Per1*, reverse, 5′-CGGTCTTGCTTCAGCACAGA-3′; *Per2*, forward, 5′-CAGCCACCCTGAAAAGGA-3′; *Per2*, reverse, 5′-GTGAGGGACACCACACTCTC-3′; *Cry1*, forward, 5′-CGGTGGAAATTGCTCTCA-3′; *Cry1*, reverse, 5′-GGCATCCTCTTCCTGACTA-3′; *Pparγ-coactivator-1α* (*Pgc-1α*), forward, 5′-ATACCGCAAAGAGCACGAGAAG-3′; *Pgc-1α*, reverse, 5′-CTCAAGAGCAGCGAAAGCGTCACAG-3′; *peroxisome proliferator activated receptor* (*Ppar*) *α*, forward, 5′-TCCCTGTTTGTGGCTGCTAT-3′; *Pparα*, reverse, 5′-TTGGGAAGAGGAAGGTGTCA-3′; *Pparγ*, forward, 5′-TGAGACCAACAGCCTGAC-3′; *Pparγ*, reverse, 5′-GGTTCACCGCTTCTTTCA-3′; *fatty acid synthase* (*Fas*), forward, 5′-TGCTCCCAGCTGCAGGC-3′; *Fas*, reverse, 5′-GCCCGGTAGCTCTGGGTGTA-3′; *sterol regulatory element-binding protein 1c* (*Srebp1c*), forward, 5′-CATGGATTGCACATTTGAAG-3′; *Srebp1c*, reverse, 5′-CCTGTGTCCCCTGTCTCA-3′; *phosphoenolepyruvate carboxykinase* (*Pepck*), forward, 5′-TTGCCTGGATGAAGTTTGAT-3′; *Pepck*, reverse, 5′-GGCATTTGGATTTGTCTTCACT-3′; *glucose-6-phosphatase* (*G6Pase*), forward, 5′-AAAGAGACTGTGGGCATCAATC-3′; *G6Pase*, reverse, 5′-AATGCCTGACAAGACTCCAGCC-3′; *β-actin*, forward, 5′-TTGTAACCAACTGGGACGATATGG-3′; *β-actin*, reverse, 5′-GATCTTGATCTTCATGGTGCTAGG-3′; *Gapdh*, forward, 5′-TGAAGGTCGGTGTGAACG-3′; and *Gapdh*, reverse, 5′-CCATTCTCGGCCTTGACT-3′.

Western blot analysis. Liver extracts were prepared using 20 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 10% glycerol, 1% Triton X-100, 1 mmol/l ethylendiaminetetraacetic acid, 2 mmol/l Na_3VO_4, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, and insoluble proteins were removed by centrifugation at 13,500 *g*. Protein content was determined employing a Bradford assay with Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA). Protein extracts were resolved by SDS-PAGE. Blots were incubated with antibody to PER1 (Santa Cruz Biotechnology, Santa Cruz, CA) and then reincubated with a secondary horseradish-conjugated antibody. Immunoreactive bands were visualized by enhanced chemiluminescence.

Statistical analysis. The results are presented as means ± SE. Two-way ANOVA followed by Bonferroni's post hoc test was used to determine variance with respect to time and mouse group (n = 6 mice/observation point for each group) (2, 21, 26). All experiments were performed at least three times to confirm that the data presented in Figs. 1–7 were reproducible.

RESULTS

CMS alters circadian mRNA expression profiles of core clock genes in the livers of BALB/c mice. First, to examine the influence of chronic stress on circadian clock gene expressions in the liver, BALB/c mice were divided into two groups: one was housed under ordinary conditions (Control-BALB) and the other was subjected to CMS (CMS-BALB) (34, 38, 39, 53). Serum parameters and hepatic gene expressions were analyzed 1 day after the last day of CMS (Fig. 1). In CMS-BALB mice, serum levels of corticosterone, a major glucocorticoid in rodents, were significantly upregulated, and their peak time

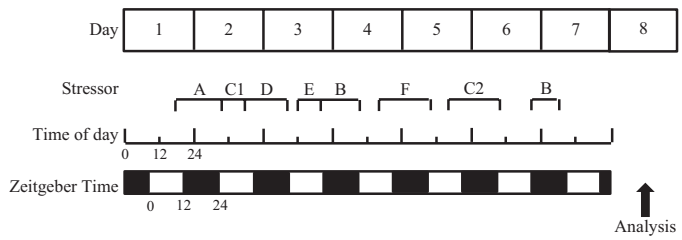


Fig. 1. Schedule of the chronic mild stress (CMS) procedure. Square brackets indicate the time that the stressor was applied to experimental mice. A, one 16-h period of water deprivation; B, two 12-h periods of continuous overnight illumination; C1, one 7-h period of 45° cage tilt; C2, one 17-h period of 45° cage tilt; D, one 17-h period in a cage without bedding chips; E, one 8-h period of food deprivation; F, one 17-h period of paired housing.

points were altered (2-way ANOVA; $F = 8.0731$, $P = 5.0 \times 10^{-4}$) (Fig. 2*A*), indicating activation and altered rhythmicity of the HPA axis in response to this CMS procedure. Body weights and serum leptin levels under CMS conditions were similar to those of the control. Blood glucose levels were also similar, whereas serum insulin levels were higher at one circadian time point in CMS-BALB (2-way ANOVA; $F = 4.2929$, $P = 1.11 \times 10^{-2}$) than in Control-BALB mice (Fig. 2*B*). The food intake amounts were measured on the day after the last day of CMS based on division into light and dark phases, but the CMS procedure did not significantly alter the food intake amount in either phase (Fig. 2*C*). To examine the hypothesis that CMS affects hepatic expressions of core clock genes, we analyzed the circadian expressions of transcripts encoding CLOCK, NPAS2, BMAL1, PER1, PER2, and CRY1 in the livers of both groups. As shown in Fig. 3, the expressions of *Npas2* (2-way ANOVA; $F = 17.0963$, $P < 1.0 \times 10^{-4}$) and *Bmal1* (2-way ANOVA; $F = 15.3862$, $P < 1.0 \times 10^{-4}$) were significantly altered at several circadian time points in CMS-BALB mice. Interestingly, rhythms of *Clock* (2-way ANOVA; $F = 19.3284$, $P < 1.0 \times 10^{-4}$), *Per1* (2-way ANOVA; $F = 30.8876$, $P < 1.0 \times 10^{-4}$), and *Cry1* (2-way ANOVA; $F = 7.4773$, $P = 6.0 \times 10^{-4}$) expressions in the liver were significantly altered in CMS-BALB mice, whereas *Per2* expression levels remained unaltered (Fig. 3*A*).

In addition, immunoblotting revealed the oscillation of PER1 protein expression. The PER1 protein levels oscillated with circadian phases, showing the highest levels at ZT 3 in CMS-BALB mice and at ZT 21 in Control-BALB mice (Fig. 3*B*). These findings are consistent with previous studies showing protein cycles to be delayed by about 6–12 h relative to the mRNA cycles, under both control and stressed conditions (13, 16). Thus, CMS altered the expression rhythms of PER1 on not only the mRNA but also the protein level in the livers of BALB/c mice.

Collectively, these findings suggest that chronic stress alters the profiles of rhythmic core clock gene expressions in the liver and generates gene-specific modulations of circadian expression patterns.

Phase alignments of the core clock genes in peripheral tissues are controlled to be expressed in a synchronized manner by the neurons of the hypothalamic SCN (6). Therefore, we next examined the core clock gene expressions in the SCN of both CMS-BALB and Control-BALB mice. Interestingly, the core clock gene expressions in the SCN showed no significant alterations in either levels or rhythmicity (Fig. 4). Thus, the central master clock is not responsible for alterations in hepatic clock gene expressions under conditions of chronic stress in BALB/c mice.

CMS does not alter circadian mRNA expression profiles of core clock genes in the livers of C57BL/6 mice. A variety of so called "zeitgebers" ("time givers"), such as food and light presence and endogenous glucocorticoid levels, are able to entrain the phase of circadian clocks (23). The CMS procedure used in this study includes water deprivation, food deprivation, and 12-h overnight illumination. In addition, as described above, this CMS procedure altered the endogenous glucocorticoid levels in BALB/c mice (Fig. 2*A*). On the other hand, C57BL/6 mice do not display activation of the HPA axis when exposed to chronic stress (24, 43). Furthermore, the same CMS procedure was confirmed to have no effect on serum cortico-

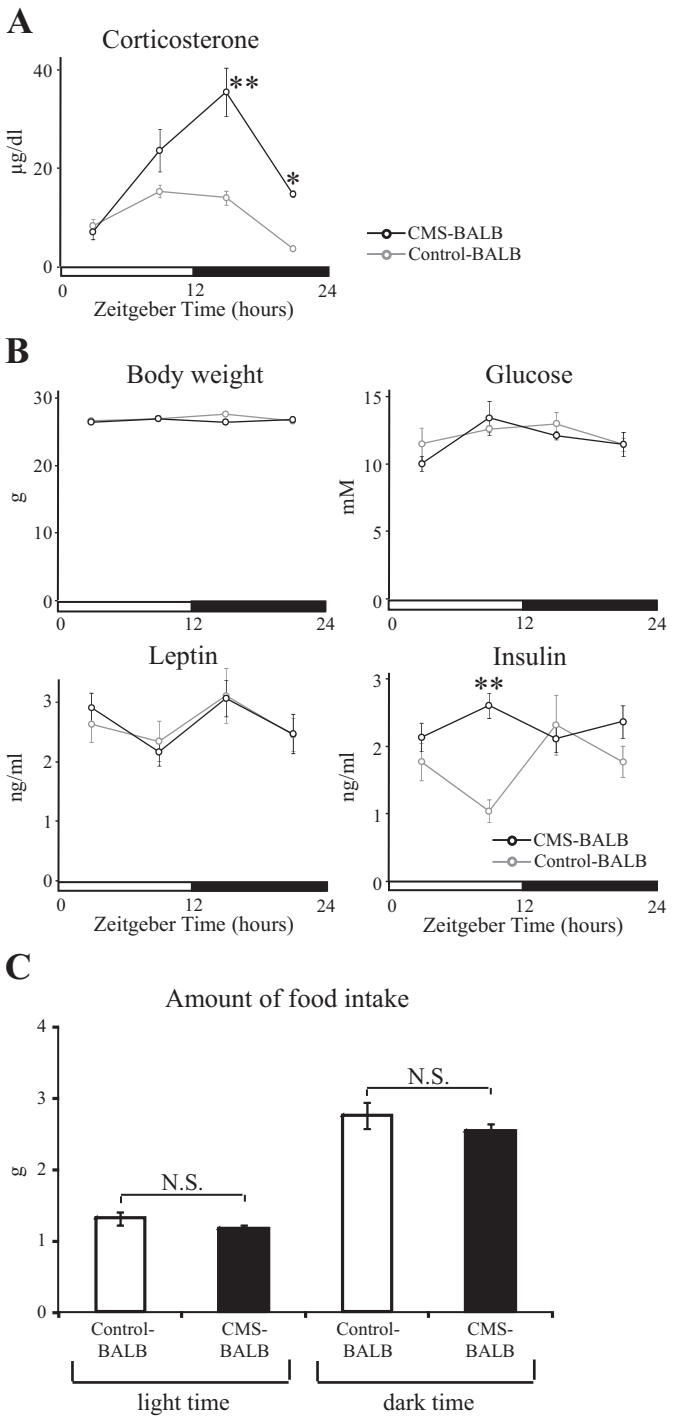


Fig. 2. Metabolic parameters of BALB/c mice. Mice were assigned to either the Control-BALB or the CMS-BALB group. Diurnal variations in plasma corticosterone (*A*), body weight, blood glucose, plasma insulin, and leptin (*B*) are shown; $n = 6$ mice/group for each observation point. Results are expressed as means ± SE. Two-way ANOVA was used to determine variance with respect to time and groups, followed by Bonferroni's post hoc test ($*P < 0.05$ and $**P < 0.01$). *C*: food intake was measured 1 day after the last day of the CMS procedure, with division into light and dark phases. Results are expressed as means ± SE. One-way ANOVA was used to determine statistical significance (N.S., not significant).

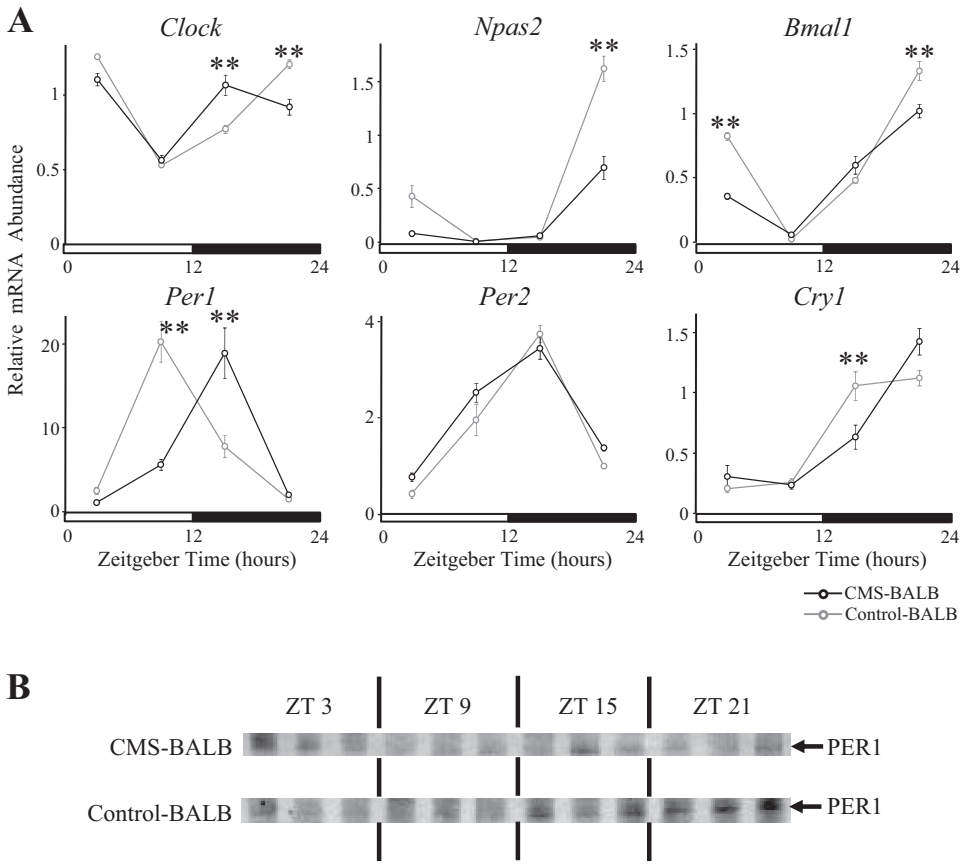


Fig. 3. Transcripts of circadian clock genes in the livers of BALB/c mice. *A*: expressions of mRNAs encoding *Clock*, *NPAS2*, *Bmal1*, *Per1*, *Per2*, and *Cry1* in the livers of Control-BALB and CMS-BALB mice; $n = 6$/group for each observation point. Results are expressed as means ± SE. Two-way ANOVA was used to determine variance with respect to time and groups, followed by Bonferroni's post hoc test (**$P < 0.01$). *B*: immunoblotting of the liver extracts of Control-BALB and CMS-BALB mice with anti-PER1 antibody.

sterone levels (38). Therefore, we examined the effects of CMS on hepatic core clock gene expressions in C57BL/6 mice.

C57BL/6 mice were divided into two groups in a manner similar to that used for BALB/c mice; one group was housed under ordinary conditions (Control-C57), and the other was subjected to the same CMS (CMS-C57). Consistent with a previous report (38), Control-C57 and CMS-C57 mice showed no significant differences in serum corticosterone levels or rhythms (Fig. 5*A*), indicating that CMS does not affect the HPA axis in these mice. Body weight, blood glucose, serum insulin, and leptin levels remained unchanged (Fig. 5*B*). No significant differences in food intake amounts were observed between CMS-C57 and Control-C57 mice during either the light or the dark phase of the day after the last day of the CMS

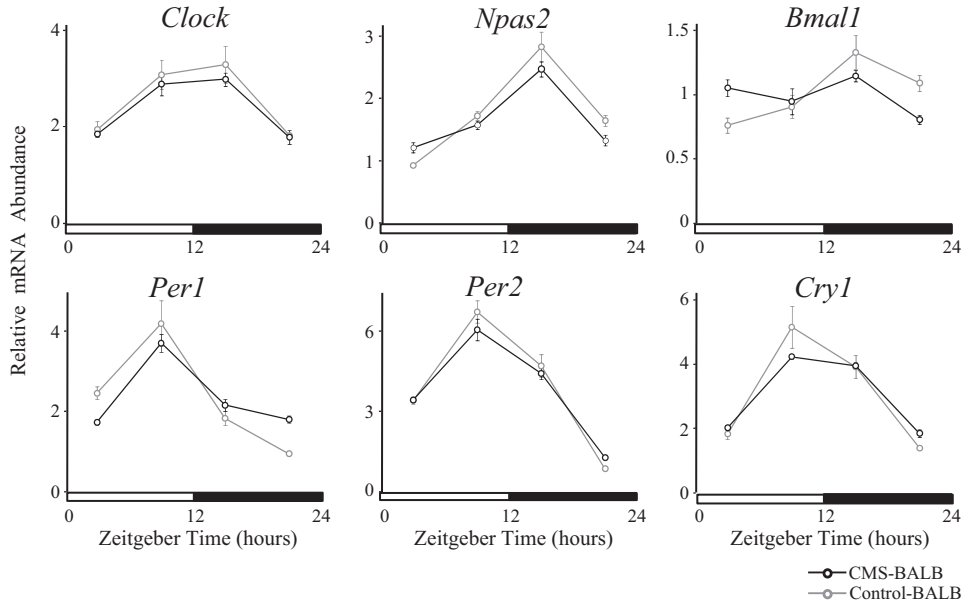


Fig. 4. Transcripts of circadian clock genes in the hypothalamic suprachiasmatic nucleus (SCN) of BALB/c mice. Expressions of mRNAs encoding *Clock*, *NPAS2*, *Bmal1*, *Per1*, *Per2*, and *Cry1* in the SCN of Control-BALB and CMS-BALB mice; $n = 6$/group for each observation point. Results are expressed as means ± SE. Two-way ANOVA was used to determine variance with respect to time and groups, followed by Bonferroni's post hoc test.

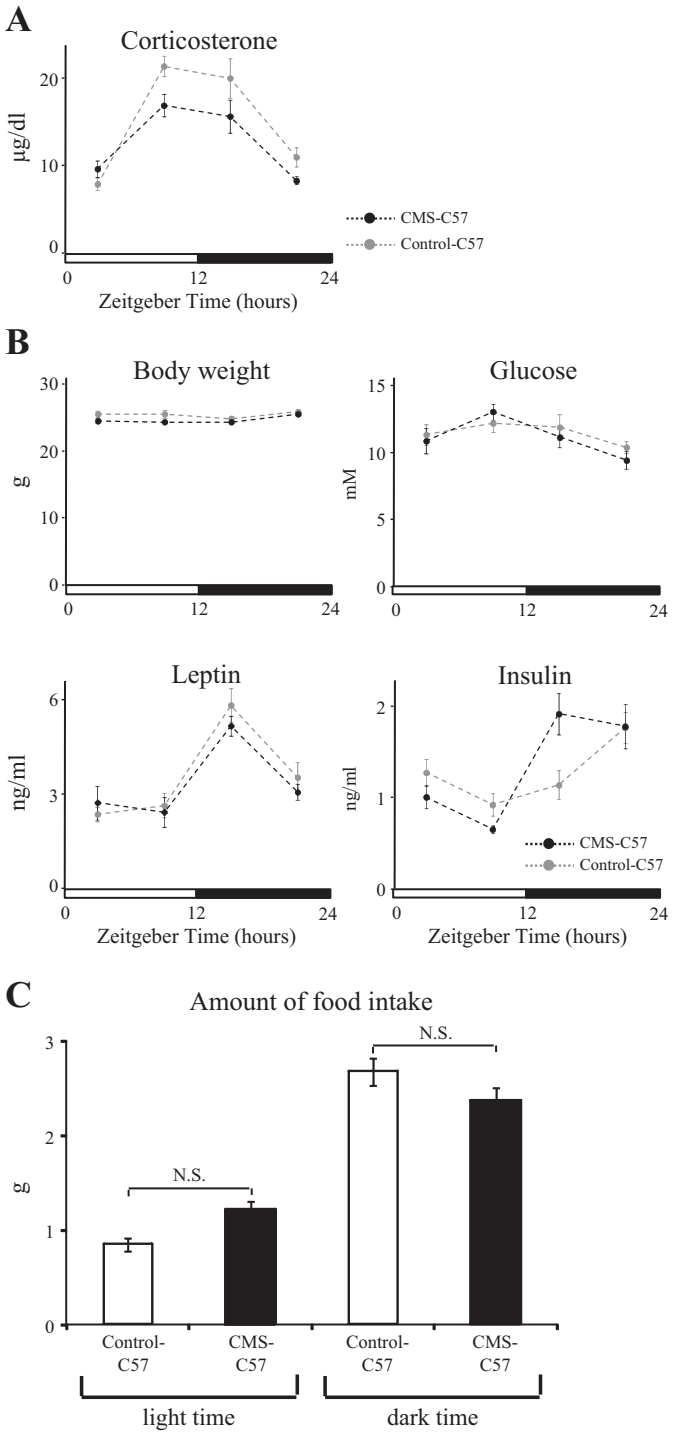


Fig. 5. Metabolic parameters of C57BL/6 mice. Mice were assigned to either the Control-C57 or the CMS-C57 group. Diurnal variations in plasma corticosterone (*A*), body weight, blood glucose, plasma insulin, and leptin (*B*) are shown; $n = 6$/group for each observation point. Results are expressed as means ± SE. Two-way ANOVA was used to determine variance with respect to time and groups, followed by Bonferroni's post hoc test. *C*: food intake was measured 1 day after the last day of the CMS procedure, with division into light and dark phases. Results are expressed as means ± SE. One-way ANOVA was used to determine statistical significance.

procedure (Fig. 5*C*). In contrast to BALB/c mice, even under CMS, the rhythms and expressions of *Clock*, *Npas2*, *Bmal1*, *Per1*, *Per2*, and *Cry1* were not significantly altered in the liver (Fig. 6). These results suggest that HPA axis activation itself, rather than each of the zeitgebers comprising the CMS procedure, plays a major role in altered circadian expressions of hepatic clock genes.

HPA axis activation alters circadian mRNA expression profiles of metabolism-related genes in the livers of BALB/c mice. Previous reports have demonstrated the liver clock to function in vivo and participate in glucose and lipid homeostasis (29, 30). Therefore, we next examined the expression rhythms of genes involved in glucose and lipid metabolism in the livers of Control-BALB, CMS-BALB, Control-C57, and CMS-C57 mice. We first analyzed the circadian expression profiles of *Pparα*, *Pparγ-1*, and *Pgc-1α*, which are involved in lipid metabolism and known to be rhythmically expressed in the liver (14). CMS significantly altered the hepatic circadian expressions of all these molecules in BALB/c (2-way ANOVA; $F = 5.7232$, $P = 3.2 \times 10^{-3}$ for *Pparα*; $F = 24.8525$, $P < 1.0 \times 10^{-4}$ for *Pparγ-1*; $F = 17.0444$, $P < 1.0 \times 10^{-4}$ for *Pgc-1α*) but not in C57BL/6 mice (Fig. 7*A*). In addition, the expression rhythm of *Pepck* (2-way ANOVA; $F = 17.2246$, $P < 1.0 \times 10^{-4}$), which is the rate-limiting enzyme in gluconeogenesis, of which expression is regulated by PGC-1α (20), was markedly altered in CMS-BALB mice (Fig. 7*B*). These results suggest an important role of HPA axis activation in the expressions of these glucose- and lipid metabolism-related genes under chronic stress conditions. In contrast, CMS altered the circadian expressions of *glucose-6-phosphatase* (*G6Pase*) in both BALB/c (2-way ANOVA; $F = 7.3724$, $P = 6.0 \times 10^{-4}$) and C57BL/6 mice (2-way ANOVA; $F = 8.6569$, $P = 3.0 \times 10^{-4}$) (Fig. 7*B*). Interestingly, this CMS procedure did not change hepatic expression profiles of *Srebp1c* and *Fas*, both of which mediate lipogenesis, in BALB/c mice, whereas their expressions were altered in C57BL/6 mice (2-way ANOVA; $F = 4.9484$, $P = 6.8 \times 10^{-3}$ for *Srebp1c*; $F = 17.5981$, $P < 1.0 \times 10^{-4}$ for *Fas*) (Fig. 7*C*). These results indicate that this CMS procedure actually affects gene expressions in the livers of C57BL/6 mice but that circadian expressions of lipogenesis-related genes in the liver are disturbed via a pathway(s) other than the HPA axis. Collectively, these findings suggest that chronic stress affects profiles of the rhythms of glucose- and lipid metabolism-related gene expressions in the liver via distinct mechanisms.

DISCUSSION

In this study, to examine whether chronic stress modifies circadian rhythms of hepatic clock genes, we chose a CMS model that has been widely used for producing a state of chronic stress (34, 38, 39, 53). This CMS procedure consists of stressors, including water deprivation, food deprivation, and 12-h overnight illumination, all of which can affect circadian expression rhythms of clock genes in peripheral tissues (11, 32). In a state of chronic stress, hepatic clock alterations were observed in BALB/c mice but not in C57BL/6 mice. These results indicate that each stressor cue by itself does not affect the rhythms of hepatic clock expressions. Rather, the modulated physiological responses to these stress cues affect hepatic oscillation. Such responses are thought to be adaptive pro-

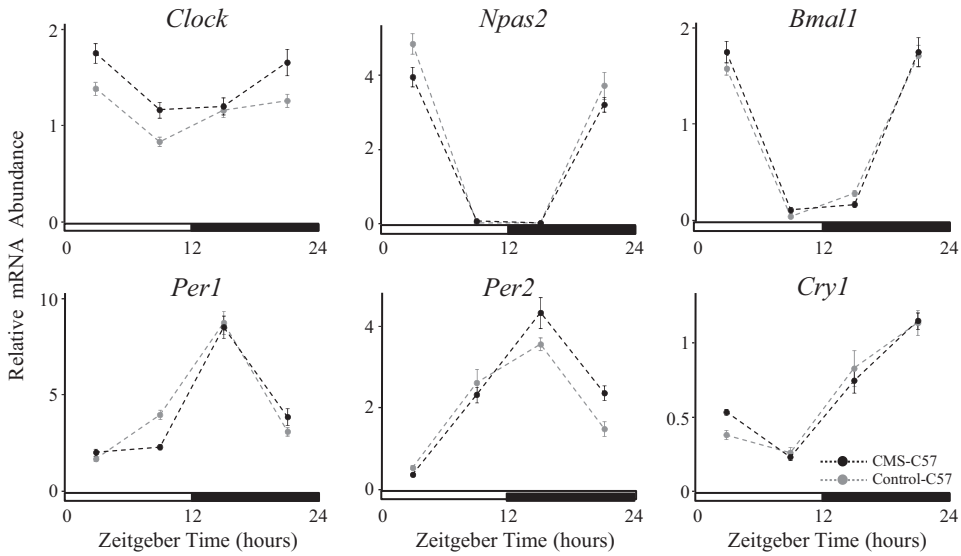


Fig. 6. Transcripts of circadian clock genes in the livers of C57BL/6 mice. Expressions of mRNAs encoding *Clock*, *NPAS2*, *Bmal1*, *Per1*, *Per2*, and *Cry1* in the livers of Control-C57 and CMS-C57 mice; $n = 6$/group for each observation point. Results are expressed as means ± SE. Two-way ANOVA was used to determine variance with respect to time and groups, followed by Bonferroni's post hoc test.

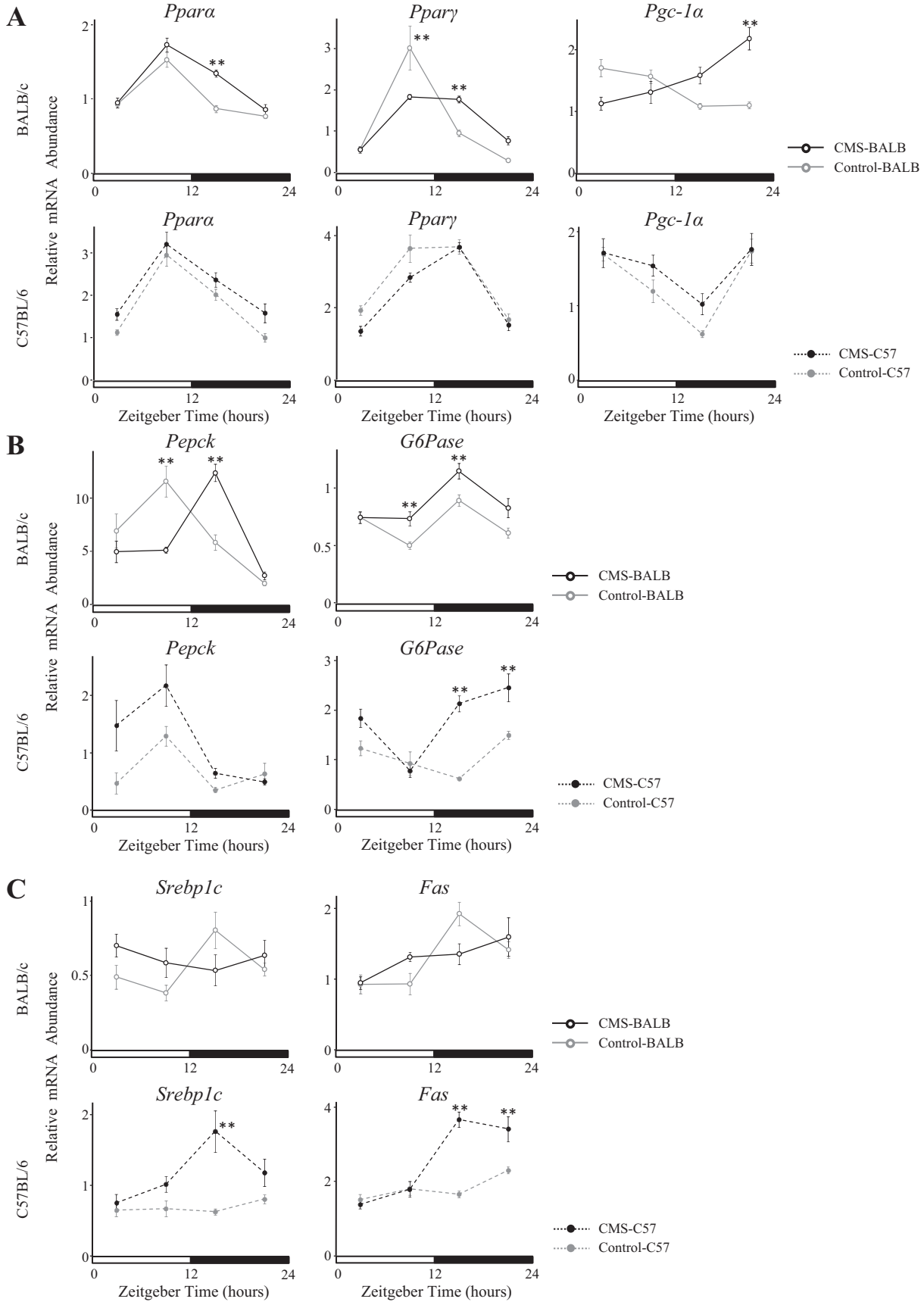
cesses that maintain homeostasis via the HPA axis and the autonomic nervous system (35). A previous study demonstrated that this CMS procedure activates the HPA axis in BALB/c mice but not in C57BL/6 mice (38). These findings are consistent with our observations that this CMS procedure raises serum corticosterone levels in BALB/c mice but not in C57BL/6 mice. On the other hand, circadian expressions of *Srebp1c* and *Fas* in the liver were remarkably changed in C57BL/6 mice, indicating that these stress cues function as zeitgebers in peripheral organs, including the liver, via a pathway other than the HPA axis. We cannot rule out the possibility that the slight, not statistically significant, suppression of corticosterone levels observed in CMS-C57 mice affects circadian expressions of these lipogenesis-related genes. However, corticosterone elevations in BALB/c mice did not significantly affect the expressions of these genes. In addition, CMS altered neither the rhythms nor the expressions of *Clock*, *Npas2*, *Bmal1*, *Per1*, *Per2*, and *Cry1* in the livers of C57BL/6 mice. In particular, given that stressed BALB/c mice displayed serum corticosterone elevations and altered circadian clock gene expressions before exhibiting any remarkable metabolic alterations in body weight and blood glucose, it is likely that overactivation of the HPA axis itself, i.e., without any metabolic disorder phenotype, is sufficient to alter the circadian clock system in response to CMS. Moreover, dysregulations of metabolism-related genes, such as *Pparα*, *Pparγ-1, Pgc-1α*, and *Pepck*, in the liver were observed in stressed BALB/c mice. These results raise the possibility that alterations in the circadian clock system are among the triggers of metabolic syndrome onset associated with chronic stress.

This CMS procedure includes 12-h overnight illumination. The SCN master clock receives direct photic input via the retinohypothalamic tract, and the presence of light is known to be the key environmental timing cue that entrains the core clock genes in the SCN (48). However, circadian expressions of these genes in the SCN were not affected by the CMS procedure in BALB/c mice. This finding further supports the importance of the HPA axis in the regulation of hepatic clock oscillation.

Recent studies indicate that the SCN affects the circadian pattern of corticosterone secretion (25). In the present study, however, CMS did not alter rhythms in the SCN. One of the plausible mechanisms whereby CMS alters the daily pattern of corticosterone secretion without altering SCN rhythms is limbic regulation of the HPA axis. Limbic regulation of the HPA axis is achieved through relaying neuronal inputs from limbic regions of the brain, such as the hippocampus and amygdala, to the paraventricular nucleus (PVN) of the hypothalamus (22). PVN releases corticotropin-releasing hormone (CRH), and CRH in turn stimulates pituitary release of adrenocorticotropic hormone (ACTH). ACTH then acts upon cells in the adrenal cortex to augment the synthesis and secretion of glucocorticoids. Therefore, CMS can alter the daily pattern of corticosterone secretion via the limbic system without involving the SCN.

It is well known that glucocorticoids, which act as end effectors of the HPA axis, reset the circadian rhythms of peripheral organs, including the liver, by shifting the phase of circadian gene expressions (7, 35). In vitro studies have demonstrated that glucocorticoids stimulate the transcriptional rhythmicities of *Per1* (5), *NPAS2* (45), *Cry1* (5), *Bmal1*, and *Clock* (36), all of which were found to show altered oscillations in the present study. Glucocorticoid receptors bind to glucocorticoids and occupy the glucocorticoid-responsive element (GRE) in the genome, thereby regulating target gene transcription (45). The GRE is located in the promoter of *Per1* and is reportedly involved in *Per1* expression (54), which allows

Fig. 7. Transcripts involved in lipid and glucose metabolism in the livers of BALB/c and C57BL/6 mice. Expressions of mRNAs encoding *peroxisome proliferator activated receptor* (*Ppar*) α, *Pparγ*, and *Pparγ-coactivator-1α* (*Pgc-1α*) (*A*), *phosphoenolepyruvate carboxykinase* (*Pepck*) and *glucose-6-phosphatase* (*G6Pase*) (*B*), and *sterol regulatory element-binding protein 1c* (*Srebp1c*) and *fatty acid synthase* (*Fas*) (*C*) in the livers of Control-BALB, CMS-BALB, Control-C57, and CMS-C57 mice; $n = 6$/group for each observation point. Results are expressed as means ± SE. Two-way ANOVA was used to determine variance with respect to time and groups, followed by Bonferroni's post hoc test (**$P < 0.01$).



glucocorticoids to directly interact with PER1. Therefore, upregulation of glucocorticoids induced by overactivation of the HPA axis might directly affect the circadian expressions of core clock genes in the liver.

Persistent stimulation of the HPA axis by various stressors causes metabolic disorders, such as insulin resistance/overt diabetes mellitus, hypertension, and dyslipidemia (7, 35). Interestingly, these metabolic disturbances are also induced by dysregulation of the clock system (6, 14). However, whether modulation of the HPA axis leads to metabolic problems through disruption of the clock system, or these systems influence the same metabolic pathway independently, has not been determined. Several molecules, such as PPARα, PPARγ and PGC-1α, play crucial roles in intermediary metabolism and respond to both the clock system and glucocorticoids (6, 35). In the liver, BMAL1 (8) and CLOCK (37) have both been demonstrated to be upstream regulators of *Pparα* gene expression. CRY1 regulates the activity of cAMP response element-binding (CREB) protein (56), and CREB in turn inhibits hepatic PPARγ (18) or enhances hepatic PGC-1α expression (19). Consistent with these reports, we observed perturbed rhythms of the expressions of core clock genes, including *Bmal1*, *Clock*, and *Cry*1, and metabolism-related genes, including *Pparα*, *Pparγ*, and *Pgc-1α*, in the livers of CMS-BALB mice. Although PER2 has been shown in adipocytes to directly repress PPARγ (15), hepatic expression rhythms of *Per2* were not altered in the present study. Therefore, altered expressions of *Per2* are not required for CMS-induced perturbation of metabolism-related gene expressions in the liver. Furthermore, rhythms of *Pepck* expressions were also affected in the livers of CMS-BALB mice. PGC-1α mediates the induction of PEPCK by glucocorticoids (19). Thus, in a chronically stressed state, the overactivated HPA axis acts together with the clock system, which may lead to the onset of metabolic disturbance in the liver.

Recent studies have suggested that rhythmic abnormalities affect energy homeostasis as well as glucose and lipid metabolism (40, 42, 50). Genetically obese strains such as KK-Ay (3) and *db*/*db* mice (28), and mice with high-fat diet-induced obesity (27), exhibit attenuated circadian expressions of several clock genes in the liver. Intriguingly, in all of these murine models, serum corticosterone levels were shown to be altered (1, 27). In addition, circadian expressions of hepatic clock genes are impaired in young *ob*/*ob* mice even before the onset of metabolic abnormalities, and this impairment in rhythmic expressions of hepatic clock genes was restored by leptin treatment (2). Serum corticosterone levels are increased in *ob*/*ob* mice (1, 52), and leptin treatment depresses corticosterone levels via suppression of the HPA axis (17). Taking these previous reports and our present results together, HPA axis activation may contribute to alterations in circadian clock gene expressions in the liver, not only under chronic stress conditions, but also in states of excess nutrition. This speculation is supported by several reports in which high fat diets exaggerated the responsiveness of the HPA axis to chronic stress (31, 49).

In summary, to our knowledge, this is the first report showing that chronic stress perturbs the circadian expressions of clock genes in the liver. Overactivaton of the HPA axis is likely to play an important role in the underlying mechanism. The dysregulation of circadian expressions of genes regulating metabolism might trigger metabolic disturbances in states of chronic stress.

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DISCLOSURES

The authors have no conflicts of interest, financial or otherwise, to declare.

AUTHOR CONTRIBUTIONS

Author contributions: K.T. T.Y. and H.K. conception and design of research; K.T. T.Y. and H.K performed experiments; K.T., T.Y., and H.K. analyzed data; K.T., T.Y., S.T., K.K., Y.S., Y.M., Y.I., J.I., K.U., Y.H., S.S., Y.O., and H.K. interpreted results of experiments; K.T. prepared figures; K.T., T.Y., and H.K. drafted manuscript; K.T., T.Y., and H.K. edited and revised manuscript; K.T., T.Y., S.T., K.K., Y.S., Y.M., Y.I., J.I., K.U., Y.H., S.S., Y.O., and H.K. approved final version of manuscript.

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