

Resetting process of peripheral circadian gene expression after the combined reversal of feeding schedule and light/dark cycle via a 24-h light period transition in rats

T. Wu, Y. Ni, F. ZHUGE, Z. Fu*

College of Biological and Environmental Engineering, Zhejiang University of Technology, China

* Corresponding author. College of Biological and Environmental Engineering, Zhejiang University of Technology, No.6 District, Zhaohui, Hangzhou, Zhejiang, 310032, China

Tel: 86-571-8832-0599

Fax: 86-571-8832-0599

e-mail: azwfu2003@yahoo.com.cn

Running title: Resetting of the peripheral clocks in mammals

Summary

To investigate the effect of light cue on the resetting of the peripheral clocks, we examined the resetting processes of clock genes (*Per1*, *Per2*, *Bmal1*, *Cry1*, *Dec1*, and *Rev-erb α*) in the liver and heart of rats after the feeding and light-dark (LD) reversal via a 24-h light period transition. The liver clock was reset quickly within 3 days, while the heart clock needed a longer time course of 5-7 days to be completely re-entrained. Moreover, the re-entrainment of *Per1* and *Per2* in the liver clock was more rapid than that of the other four clock genes, suggesting the important role of these two clock genes in initiating the circadian resetting of the hepatic clock. However, the resetting rates of these two clock genes were as similar as the others in the heart clock. Therefore, the resetting mechanisms underlining these two peripheral clocks may be totally distinct. Furthermore, the re-entrainment of the liver and heart clocks were relatively lengthened after the feeding and LD reversal via a light period transition compared to a dark period transition, suggesting a simultaneous shift of feeding schedule and the LD cycle may facilitate the circadian resetting in rats.

Keywords: light/dark reversal, peripheral clocks, liver, heart

Introduction

In mammals, the circadian rhythms in behaviour and physiology are organized by a central pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus and suboscillators in peripheral tissues such as the liver, heart, etc (Green *et al.* 2008). One of the essential features of the circadian clock is its entrainability to daily changes in the environment. The SCN clock is reset mainly by the light cue via the retinohypothalamic tract (Barrett and Takahashi 1995, Dunlap 1999, Hastings 1997), whereas the peripheral clocks seem to be reset mainly by the food cue in mammals (Damiola *et al.* 2000, Hara *et al.* 2001, Stokkan *et al.* 2001, Wakamatsu *et al.* 2001). To date, numerous studies have attempted to explain mechanisms of the light induced resetting of the SCN clock. For example, light pulses applied during the night lead to increased levels of *Per1* and *Per2* in the SCN that may be important to the light-induced circadian resetting of the SCN clock (Albrecht *et al.* 1997, Liu *et al.* 1997, Shigeyoshi *et al.* 1997). Moreover, Ishida *et al.* (2005) found that light could rapidly induce *Per1* expression in the adrenal gland via the SCN-sympathetic nervous system. However, the effect of light cue on the re-entrainment of the food dominated peripheral clocks has not been fully discussed.

In previous studies on the light-induced circadian resetting, it was demonstrated that phase delays are accomplished more rapidly and with less disturbance than phase advances (Illnerova *et al.* 1987, Miranda-Anaya *et al.* 2000, Reddy *et al.* 2002, Sujino *et al.* 2007, Takamura *et al.* 1991). However, the discrepancy between the circadian resetting induced by photoperiod alteration via dark or light period transition has

seldom been investigated. In the light dominated pineal clock (Wu *et al.* 2008a), our recent studies indicated that the resetting processes of clock genes were noticeably lengthened after the light-dark (LD) reversal via a light period transition as compared with that via a dark period transition (Wu *et al.* 2009). Although the circadian phases of clock genes in other peripheral tissues are mainly dominated by food cues (Damiola *et al.* 2000, Hara *et al.* 2001, Stokkan *et al.* 2001, Wakamatsu *et al.* 2001), we also found that reversing the feeding schedule together with the LD cycle via a dark period transition markedly enhanced the feeding-induced resetting of peripheral clocks (Wu *et al.* 2008b). Thus, we are further interested in whether the food dominated resetting processes of clock genes in peripheral tissues would be any different after the LD reversal via a light period transition as compared with that via a dark period transition in our previous studies (Wu *et al.* 2008b). To clarify further, we investigated the resetting processes of *Per1* and *Per2*, as well as other clock genes (*Bmal1*, *Cry1*, *Dec1*, *Rev-erba*) in the liver and heart of rats after the reversal of feeding schedule and LD cycle via a 24-h light period transition.

Methods

Animals and Experimental Design

Male Wistar rats (body weight: 90 to 100 g) were kept in our animal facilities (illumination with fluorescent strip lights of 200 lux at cage level; 22±1°C) under a 12 h : 12 h light-dark (LD) cycle. Water was available *ad libitum*, while food was provided only in the dark period. The onset of light was defined as *Zeitgeber* time 0

(ZT0), and the onset of darkness was ZT12.

To test the resetting processes of clock genes in the peripheries, rats were divided into two groups after their adaptation to the normal LD cycle. In the control group, rats were sacrificed at 4-h intervals of the daily cycle, starting at ZT0. In the resetting group, rats were starved for two days and then subjected to the reversal conditions for both feeding schedule and LD cycle by extending the light period for 24 hours. Rats in this group were sampled every 4 h from the start of the phase change on the 1st day (Time 0–24 h), the 3rd day (Time 48–72 h), the 5th day (Time 96–120 h), and the 7th day (Time 144–168 h). The purpose of fasting was to make the rats take food immediately after the food phase shift, allowing the information of food stimuli to be transmitted immediately as the feeding schedule changes. We have confirmed that fasting for 2 days did not alter the expression of circadian genes in the liver and heart (Dong *et al.* 2009).

All rats were killed after deep anesthesia by an i.p. injection of pentobarbital sodium. Livers and hearts were quickly removed, immediately frozen on dry ice and kept at -80°C. During the dark phase, the dissection was carried out under dim red light. Every effort was made to minimize animal suffering and the number of rats required for each experiment. All animal care and use procedures were in accordance with guidelines of the Research Committee of Zhejiang University of Technology.

RNA Isolation and Reverse Transcription

The total RNA from livers and hearts was isolated using the TRIzol reagent

(Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with some modifications as previously described (Wu *et al.* 2008b).

Real-Time PCR

Quantitative real time PCR was performed using the SYBR[®] ExScript[™] PCR Kit (Takara Biochemicals, China) in a total volume of 10 μ L. The primer sequences for *Per1*, *Per2*, *Bmal1*, *Cry1*, *Dec1* were the same as in our previous report (Wu *et al.* 2008a). The primer sequences for *Rev-erba* were as follows: Forward, ACAGCTGACACCACCCAGATC; Reverse, CATGGGCATAGGTGAAGATTTCT. The PCR amplification and quantification were performed in a real-time PCR system (ABI-7300, USA) as follows: denaturation for 10 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, 31 s at 60 °C. The relative quantification of gene expression was analyzed from the measured threshold cycles (C_t) by using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). The data were normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Data Analysis

All data are given as the mean \pm SEM of n=4 animals. The values for the mRNA levels are presented as relative values in all experiments. The daily oscillation of each circadian gene was analyzed by one-way ANOVA. The effect of group on the expression pattern of each gene was tested by two-way ANOVA. When a significant difference was observed by using two-way ANOVA, the difference at each time-point

was analyzed by the Student-Newman-Keuls test, and was considered statistically significant when p was less than 0.05 or 0.01 ($*p < 0.05$; $**p < 0.01$). Moreover, we estimated the peak phase of each cycling gene from the peak time of the most highly correlated cosine wave (Cosine Wave Analysis). The mRNA levels were fitted in Matlab 7.0.1 program by a nonlinear least-squares regression with the following cosine-wave equation (cosinor): $y = [A+B \times \cos (2\pi \times (T - C)/24)]$ where y represented the level of mRNA, A was the mean level of mRNA, B was the amplitude of mRNA oscillation, C was the acrophase of mRNA oscillation, and T was the time (hr). The peak time was considered when the mRNA level at a given time-point was within 95% confidence bounds of the expected acrophase. The value R^2 quantified goodness of fit. Statistical significance of the cosine fit was considered when F was greater than $F_{0.05}$ ($*F > F_{0.05}$).

Results

Resetting processes of clock genes in the liver after the reversal of feeding schedule and light-dark cycle via a 24-h light period transition

To determine the effect of the LD reversal on the feeding-induced resetting of clock genes in the liver, we first examined the 24-h expression patterns of clock genes (*Per1*, *Per2*, *Bmal1*, *Cry1*, *Dec1*, *Rev-erba*) in the control condition, and then in the 1st, 3rd, and 5th days after the LD reversal via a 24-h light period transition (Fig. 1). Expression profiles of all selected clock genes exhibited a clear daily rhythmicity in the liver of the control rats (Table 1, $p < 0.05$; Table 2, $F > F_{0.05}$). After the reversal of

feeding schedule and LD cycle, we compared the expression profiles of all selected genes using two-way ANOVA tests for the effect of group, time and their interaction (Table 1), which revealed a group \times time interaction for all examined genes in every resetting days except for *Bmal1* in day 1. *Per1* and *Per2* displayed a rapid phase-shift rate (Table 2). In day 1, these two clock genes did not display a significant circadian rhythm, respectively (Table 2, $F < F_{0.05}$). In day 3, the peak phases of both two clock genes were shifted 12 h, and the newly established peak phases were well remained on day 5 (Table 2). The resetting processes of other four clock genes were prolonged compared to that of *Per1* and *Per2* (Table 2). In day 1, *Bmal1*, *Cry1*, and *Dec1* showed a small peak phase shift that was not more than 4 h, while *Rev-erba* did not display any significant circadian rhythm (Table 2, $F < F_{0.05}$). Then, larger phase shift was displayed for each clock gene in day 3 (Table 2). Finally, the circadian phases of these four clock genes were totally reversed on day 5 (Table 2).

Resetting processes of clock genes in the heart after the reversal of feeding schedule and light-dark cycle via a 24-h light period transition

In comparison to the liver clock, we then monitored the expression profiles of *Per1* and *Per2* as well as other clock genes (*Bmal1*, *Cry1*, *Dec1*, *Rev-erba*) in the heart in the same selected days as described above (Fig. 2). Expression profiles of these clock genes also exhibited a clear daily rhythmicity in the heart of the control rats (Table 3, $p < 0.05$; Table 4, $F > F_{0.05}$). After the reversal of feeding schedule and LD cycle, we compared the expression profiles of these genes using two-way ANOVA tests for the

effect of group, time and their interaction (Table 3), which revealed a group \times time interaction for all examined genes in every resetting days. As for *Per1* and *Per2*, the resetting processes of these two clock genes were prolonged in the heart compared to that in the liver. The 12-h peak phase shift of *Per1* and *Per2* was not accomplished until day 5 (Table 4). And the established peak phases of *Per1* and *Per2* in day 5 were remained in day 7 (Table 4). With regard to the other examined clock genes (*Bmall*, *Cry1*, *Dec1*, *Rev-erb α*), 5-7 days were needed to completely reverse the circadian phases of these clock genes in the heart (Table 4). Moreover, the resetting course was also characterized with gene-specific phase shift rates that were distinct to that in the liver. *Bmall*, *Dec1*, and *Rev-erb α* displayed a relatively rapid phase shift rate by completing the resetting of their circadian phases within 5 days, while the circadian phase of *Cry1* was not completely reset until day 7 (Table 4).

Discussion

In the present study, we found that an abrupt 12-h phase shift of feeding schedule and LD cycle entrains the peripheral clocks with gene- and tissue- specific circadian resetting properties. The examined clock genes (*Per1*, *Per2*, *Bmall*, *Cry1*, *Dec1*, and *Rev-erb α*) finished their re-entrainment within 3-5 days in the liver, while their resetting courses took 5-7 days in the heart. Therefore, the circadian resetting proceeds faster in the liver than in the heart after the 12-h phase shift of feeding schedule and LD cycle, which is in accordance with the previous studies on the feeding-induced circadian resetting of peripheral clocks (Damiola *et al.* 2000, Wu *et al.* 2008b). The food cue is a principal timing cue for peripheral clocks, which can

directly stimulate the liver clock rather than the heart clock. While the light-dark cycle can only indirectly entrain the circadian rhythm of the peripheral clocks. Therefore, the differential resetting rates of the liver and heart clocks may be mainly induced by the feeding stimulus. In addition, our recent studies reported that the resetting processes of *Bmal1*, *Cry1*, *Per1*, and *Dec1* after the same 12-h phase shift of LD cycle and feeding schedule via a 24-h light period transition were completed within 7-9 days in the rat pineal gland (Wu *et al.* 2009). Thus, the circadian clock system displayed differential resetting rates of peripheral clocks after the 12-h phase shift of feeding schedule and LD cycle. Further understanding of the discrepancy of circadian resetting in multiple peripheral clocks may provide new knowledge to the molecular mechanism of jet lag.

The examined clock genes also displayed dissimilar resetting sequences between the liver and heart clocks. Since the resetting of circadian clocks to external time cues is initiated with rapid response of clock components, identification of resetting sequences of clock genes may be useful to explore the specific roles of different clock genes in the circadian re-entrainment. The light-induced acute expression of *Per1* and *Per2* most probably plays an important role in the resetting of the SCN clock (Albrecht *et al.* 1997, Liu *et al.* 1997, Shigeyoshi *et al.* 1997), because light-dependent phase-advance or delay of the clock is impaired in mice deficient in *Per1* and *Per2* (Albrecht *et al.* 2001). In the present study, *Per1* and *Per2* were rapidly reset within 3 days, while the other four clock genes *Bmal1*, *Cry1*, *Dec1* and *Rev-erba* were not completely re-entrained until day 5 in the liver clock (Table 2).

This observation suggested that *Per1* and *Per2* may also be important in initiating the circadian resetting of the liver clock, which was consistent with the observation in the adrenal gland (Ishida *et al.*, 2005). The relative slow resetting of *Bmal1*, *Cry1*, *Dec1* and *Rev-erba* might result from a shift in the circadian feedback loops. In contrast, the time course of *Per1* and *Per2* were as similar as the other examined clock genes in the heart clock, which were mostly reset within 5 days (Table 4). This result suggested that the resetting mechanisms of the liver and heart clocks may be distinct. Moreover, *Per1* and *Per2* were considered to be sensitive to external time cues. Recently, a lack of food anticipation is specifically associated with a mutation of *Per2*, demonstrating the critical involvement of this gene in receiving the feeding stimulus (Feillet *et al.*, 2006). In the present study, only the liver clock was directly stimulated by the food cue, suggesting that the rapid resetting of *Per1* and *Per2* may be induced by the feeding stimulus. Studies on the feeding-induced rapid resetting of the circadian clock in the liver may be useful to give an further interpretation.

We found that the resetting processes of clock genes in the liver and heart after the feeding and LD reversal via a light period transition in the present study were different from that via a dark period transition in our previous studies (Wu *et al.* 2008b). In the liver clock, *Bmal1*, *Per1*, *Cry1* and *Dec1* were almost reset within 2 days after the LD reversal via a dark period transition, but they needed 3-5 days to completely reverse their circadian phases via a light period transition (Table 2). Similarly, these four clock genes displayed relatively rapid phase shift rates by completing their circadian resetting within 3-5 days in the heart after the LD reversal

via a dark period transition. In the present study, 5-7 days were needed for these genes to be completely re-entrained in the heart (Table 4). Thus, the resetting processes of clock genes in the liver and heart are obviously lengthened after the phase shift of feeding schedule and LD cycle via a light period transition compared to a dark period transition, which is consistent with our previous reports in the pineal gland (Wu *et al.* 2009). The mechanism by which the peripheral clocks differently responded to the LD reversal via a light or dark period transition is currently unknown. The different feeding and lighting regime in the transition period may be an important inducement. When the LD reversal was accomplished via a dark period transition, the food cue stimulated the animals as soon as the LD reversed. However, when the LD reversal was accomplished via a light period transition, the food cue affected the animals 12 h after the LD reversion. Therefore, the simultaneous stimulus of light and food cues might be more effective to entrain the circadian rhythm of peripheral clocks.

In conclusion, the resetting processes of clock genes in the liver and heart were characterized with tissue- and gene-specific phase shift rates and resetting sequences. The examined clock genes were more rapidly reset in the liver than that in the heart. The rapid re-entrainment of *Per1* and *Per2* in the liver suggested the important role of these two clock genes in initiating the circadian resetting of this clock. Moreover, our findings indicated that the re-entrainment of the liver and heart clocks were obviously lengthened after the phase shift of LD cycle via a light period transition compared to a dark period transition, which may provide new knowledge to reduce the symptoms of jet lag.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by a grant from the Program for Changjiang Scholars and Innovative Research Teams in University (No. IRT 0653), the Natural Science Foundation of Zhejiang Province, China (No.Y204240).

References

- ALBRECHT U, SUN ZS, EICHELE G, LEE CC: A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. *Cell* **91**: 1055-1064, 1997.
- ALBRECHT U, ZHENG B, LARKIN D, SUN ZS, LEE CC: MPer1 and mper2 are essential for normal resetting of the circadian clock. *J Biol Rhythms* **16**: 100-104, 2001.
- BARRETT RK, TAKAHASHI JS: Temperature compensation and temperature entrainment of the chick pineal cell circadian clock. *J Neurosci* **15**: 5681-5692, 1995.
- DAMIOLA F, LE MINH N, PREITNER N, KORNMANN B, FLEURY-OLELA F, SCHIBLER U. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev* **14**: 2950-2961, 2000.
- DONG Y, WU T, NI Y, KATO H, FU Z. Effect of fasting on the peripheral circadian gene expression in rats. *Biological Rhythm Research* doi: 10.1080/09291010902863289, 2009.
- DUNLAP JC: Molecular bases for circadian clocks. *Cell* **96**: 271-290. 1999.
- FEILLET CA, RIPPERGER JA, MAGNONE MC, DULLOO A, ALBRECHT U, CHALLET E. Lack of food anticipation in *Per2* mutant mice. *Curr Biol* **16**: 2016-2022, 2006
- GREEN CB, TAKAHASHI JS, BASS J: The meter of metabolism. *Cell* **134**: 728-742,

2008.

HARA R, WAN K, WAKAMATSU H, AIDA R, MORIYA T, AKIYAMA M, SHIBATA S. Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus. *Genes Cells* **6**: 269-278, 2001.

HASTINGS MH: Circadian clocks. *Curr Biol* **7**: R670-672, 1997.

ILLNEROVA H, VANECEK J, HOFFMANN K: Adjustment of the rat pineal N-acetyltransferase rhythm to eight-hour shifts of the light-dark cycle: advance of the cycle disturbs the rhythm more than delay. *Brain Res* **417**: 167-171, 1987.

ISHIDA A, MUTOH T, UEYAMA T, BANDO H, MASUBUCHI S, NAKAHARA D, TSUJIMOTO G, OKAMURA H. Light activates the adrenal gland: timing of gene expression and glucocorticoid release. *Cell Metab* **2**: 297-307, 2005.

LIU C, WEAVER DR, Jin X, SHEARMAN LP, PIESCHL RL, GRIBKOFF VK, REPPERT SM: Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. *Neuron* **19**: 91-102, 1997.

LIVAK KJ, SCHMITTGEN TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. *Methods* **25**: 402-408, 2001.

MIRANDA-ANAYA M, BARTELL PA, YAMAZAKI S, MENAKER M: Circadian rhythm of ERG in Iguana iguana: role of the pineal. *J Biol Rhythms* **15**: 163-171, 2000.

REDDY AB, FIELD MD, MAYWOOD ES, HASTINGS MH: Differential

resynchronisation of circadian clock gene expression within the suprachiasmatic nuclei of mice subjected to experimental jet lag. *J Neurosci* **22**: 7326-7330, 2002.

SHIGEYOSHI Y, TAGUCHI K, YAMAMOTO S, TKEKIDA S, YAN L, TEI H, MORIYA T, SHIBATA S, LOROS JJ, DUNLAP JC, OKAMURA H: Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. *Cell* **91**: 1043-1053, 1997.

STOKKAN KA, YAMAZAKI S, TEI H, SAKAKI Y, MENAKER M: Entrainment of the circadian clock in the liver by feeding. *Science* **291**: 490-493, 2001.

SUJINO M, NAGANO M, FUJIOKA A, SHIGEYOSHI Y, INOUYE ST: Temporal profile of circadian clock gene expression in a transplanted suprachiasmatic nucleus and peripheral tissues. *Eur J Neurosci* **26**: 2731-2738, 2007.

TAKAMURE M, MURAKAMI N, TAKAHASHI K, KURODA H, ETOH T: Rapid reentrainment of the circadian clock itself, but not the measurable activity rhythms to a new light-dark cycle in the rat. *Physiol Behav* **50**: 443-449, 1991.

WAKAMATSU H, YOSHINOBU Y, AIDA R, MORIYA T, AKIYAMA M, SHIBATA S: Restricted-feeding-induced anticipatory activity rhythm is associated with a phase-shift of the expression of mPer1 and mPer2 mRNA in the cerebral cortex and hippocampus but not in the suprachiasmatic nucleus of mice. *Eur J Neurosci* **13**: 1190-1196, 2001.

WU T, JIN Y, KATO H, FU Z: Light and food signals cooperate to entrain the rat pineal circadian system. *J Neurosci Res* **86**: 3246-3255, 2008a

WU T, JIN Y, NI Y, ZHANG D, KATO H, FU Z: Effects of light cues on re-entrainment of the food-dominated peripheral clocks in mammals. *Gene* **419**: 27-34, 2008b.

WU T, DONG Y, YANG Z, KATO H, NI Y, FU Z: Differential resetting process of circadian system in rat pineal gland after the reversal of light/dark cycle via a 24-h light or dark period transition. *Chronobiology International* **26**: x-y, 2009.

In press

Table 1. Interaction between group and time on clock gene transcription in the liver after the reversal of feeding schedule and LD cycle.

Gene	Days	Group	Time	Group × Time
<i>Per1</i>	Day 1	F=1.024;p=0.317	F=19.064;p<0.001	F=7.940;p<0.001
	Day 3	F=24.372;p<0.001	F=8.008;p<0.001	F=20.119;p<0.001
	Day 5	F=89.279;p<0.001	F=11.561;p<0.001	F=29.344;p<0.001
<i>Per2</i>	Day 1	F=14.274;p<0.001	F=19.923;p<0.001	F=5.380;p<0.001
	Day 3	F=106.977;p<0.001	F=16.554;p<0.001	F=43.136;p<0.001
	Day 5	F=0.101;p=0.752	F=2.683;p=0.027	F=20.916;p<0.001
<i>Rev-erbα</i>	Day 1	F=0.846;p=0.363	F=29.171;p<0.001	F=8.039;p<0.001
	Day 3	F=0.632;p=0.432	F=7.522;p<0.001	F=22.852;p<0.001
	Day 5	F=7.549;p=0.009	F=4.365;p=0.002	F=26.653;p<0.001
<i>Bmal1</i>	Day 1	F=18.290;p<0.001	F=65.358;p<0.001	F=1.373;p=0.249
	Day 3	F=2.013;p=0.164	F=21.401;p<0.001	F=24.840;p<0.001
	Day 5	F=13.778;p=0.001	F=6.540;p<0.001	F=53.125;p<0.001
<i>Cry1</i>	Day 1	F=3.761;p=0.061	F=47.346;p<0.001	F=6.507;p<0.001
	Day 3	F=0.112;p=0.740	F=8.466;p<0.001	F=40.141;p<0.001
	Day 5	F=6.813;p=0.013	F=3.014;p=0.017	F=24.806;p<0.001
<i>Dec1</i>	Day 1	F=4.628;p=0.038	F=10.355;p<0.001	F=3.152;p=0.013
	Day 3	F=2.410;p=0.129	F=2.818;p=0.022	F=9.263;p<0.001
	Day 5	F=0.229;p=0.635	F=2.426;p=0.044	F=21.057;p<0.001

Table 2. Cosine Wave Analysis of circadian gene expression in the liver clock.

Gene	Days	Acrophase time	F/ F _{0.05}	R ²	Phase shift
<i>Per1</i>	Con.	ZT0	12.86/6.61*	0.72	0 h
	Day 1	ns	1.84/6.61	0.27	ns
	Day 3	ZT12	12.36/6.61*	0.71	12 h
	Day 5	ZT12	6.97/6.61*	0.58	12 h
<i>Per2</i>	Con.	ZT4	19.51/6.61*	0.80	0 h
	Day 1	ns	1.32/6.61	0.21	ns
	Day 3	ZT16	18.39 /6.61*	0.79	12 h
	Day 5	ZT16	25.95 /6.61*	0.84	12 h
<i>Rev-erba</i>	Con.	ZT0	50.20/6.61*	0.91	0 h
	Day 1	ns	4.55/6.61	0.48	ns
	Day 3	ZT8	31.03/6.61*	0.86	8 h
	Day 5	ZT12	21.96/6.61*	0.81	12 h
<i>Bmal1</i>	Con.	ZT16	174.03/6.61*	0.97	0 h
	Day 1	ZT12~ZT16	338.18/6.61*	0.99	0~4 h
	Day 3	ZT20	79.69/6.61*	0.94	4 h
	Day 5	ZT4	36.77/6.61*	0.88	12 h
<i>Cry1</i>	Con.	ZT8	24.77/6.61*	0.83	0 h
	Day 1	ZT8~ZT12	199.53/6.61*	0.98	0~4 h
	Day 3	ZT16~ZT20	63.65/6.61*	0.93	8~12 h
	Day 5	ZT20	25.71/6.61*	0.84	12 h
<i>Dec1</i>	Con.	ZT4	39.59/6.61*	0.89	0 h
	Day 1	ZT0	9.95/6.61*	0.67	4 h
	Day 3	ZT12~ZT16	16.93/6.61*	0.77	8~12 h
	Day 5	ZT16	13.38/6.61*	0.73	12 h

*Statistical significance of the cosine fit was considered when F was more than $F_{0.05}$ ($F > F_{0.05}$); Acrophase time was considered when the mRNA level at a given time-point was within 95% confidence bounds of the acrophase; The value R^2 quantified goodness of fit; Phase shift was the time lag between the acrophase time in the control condition and that in resetting days; ns, no significant 24-h rhythm.

Table 3. Interaction between group and time on clock gene transcription in the heart after the reversal of feeding schedule and LD cycle.

Gene	Days	Group	Time	Group \times Time
<i>Per1</i>	Day 1	F=11.661;p=0.002	F=11.841;p<0.001	F=3.312;p=0.010
	Day 3	F=15.487;p<0.001	F=5.761;p=0.001	F=11.151;p<0.001
	Day 5	F=8.036;p=0.007	F=5.835;p<0.001	F=19.904;p<0.001
	Day 7	F=12.453;p=0.001	F=4.649;p=0.001	F=7.481;p<0.001
<i>Per2</i>	Day 1	F=12.801;p=0.001	F=20.065;p<0.001	F=8.211;p<0.001
	Day 3	F=0.018;p=0.893	F=8.497;p<0.001	F=16.055;p<0.001
	Day 5	F=33.516;p<0.001	F=11.003;p<0.001	F=32.971;p<0.001
	Day 7	F=3.538;p=0.067	F=19.496;p<0.001	F=15.140;p<0.001
<i>Rev-erba</i>	Day 1	F=7.973;p=0.007	F=39.210;p<0.001	F=8.573;p<0.001
	Day 3	F=5.779;p=0.021	F=16.465;p<0.001	F=20.575;p<0.001
	Day 5	F=9.111;p=0.004	F=9.315;p<0.001	F=43.105;p<0.001
	Day 7	F=1.884;p=0.178	F=10.450;p<0.001	F=38.923;p<0.001
<i>Bmal1</i>	Day 1	F=100.491;p<0.001	F=157.902;p<0.001	F=23.944;p<0.001
	Day 3	F=65.152;p<0.001	F=54.631;p<0.001	F=29.756;p<0.001

	Day 5	F=180.761;p<0.001	F=26.836;p<0.001	F=122.038;p<0.001
	Day 7	F=64.953;p<0.001	F=10.264;p<0.001	F=65.270;p<0.001
<i>Cry1</i>	Day 1	F=28.279;p<0.001	F=17.434;p<0.001	F=4.414;p=0.002
	Day 3	F=5.490;p=0.024	F=12.363;p<0.001	F=8.444;p<0.001
	Day 5	F=1.750;p=0.193	F=5.739;p<0.001	F=20.072;p<0.001
	Day 7	F=5.974;p=0.019	F=4.127;p=0.003	F=21.860;p<0.001
<i>Dec1</i>	Day 1	F=0.444;p=0.509	F=22.759;p<0.001	F=4.976;p=0.001
	Day 3	F=0.210;p=0.649	F=14.500;p<0.001	F=14.852;p<0.001
	Day 5	F=7.581;p=0.009	F=2.645;p=0.029	F=22.743;p<0.001
	Day 7	F=3.274;p=0.078	F=4.012;p=0.003	F=40.155;p<0.001

Table 4. Cosine Wave Analysis of circadian gene expression in the heart clock

Gene	Days	Acrophase time	F/F _{0.05}	R ²	Phase shift
<i>Per1</i>	Con.	ZT0	14.63/6.61*	0.75	0 h
	Day 1	ns	3.36/6.61	0.40	ns
	Day 3	ns	4.84/6.61	0.49	ns
	Day 5	ZT12	20.73/6.61*	0.84	12 h
	Day 7	ZT12	150.48/6.61*	0.97	12 h
<i>Per2</i>	Con.	ZT0	17.51/6.61*	0.78	0 h
	Day 1	ZT4	52.09/6.61*	0.91	4 h
	Day 3	ns	1.12/6.61	0.18	ns
	Day 5	ZT12	36.77/6.61*	0.88	12 h
	Day 7	ZT12	32.8/6.61*	0.87	12 h
<i>Rev-erba</i>	Con.	ZT20	8.14/6.61*	0.62	0 h
	Day 1	ZT20~ZT24	16.30/6.61*	0.77	0~4 h

	Day 3	ZT0~ZT4	9.11/6.61*	0.65	4~8 h
	Day 5	ZT8	75.93/6.61*	0.94	12 h
	Day 7	ZT8	27.62/6.61*	0.85	12 h
<i>Bmall</i>	Con.	ZT12	17.53/6.61*	0.78	0 h
	Day 1	ZT12~ZT16	154.07/6.61*	0.97	0~4 h
	Day 3	ZT16~ZT20	14.51/6.61*	0.74	4~8 h
	Day 5	ZT0	121.6/6.61*	0.96	12 h
	Day7	ZT0	46.9/6.61*	0.90	12 h
<i>Cry1</i>	Con.	ZT12	14.5/6.61*	0.74	0 h
	Day 1	ZT8~ZT12	142.24/6.61*	0.97	0~4 h
	Day 3	ZT12~ZT16	14.45/6.61*	0.74	0~4 h
	Day 5	ZT20	45.96/6.61*	0.90	8 h
	Day 7	ZT0	46.98/6.61*	0.90	12 h
<i>Decl</i>	Con.	ZT20	39.85/6.61*	0.89	0 h
	Day 1	ZT0	31.54/6.61*	0.86	4 h
	Day 3	ZT0~ZT4	13.01/6.61*	0.72	4~8 h
	Day 5	ZT8	54.84/6.61*	0.92	12 h
	Day 7	ZT8	85.98/6.61*	0.95	12 h

*Statistical significance of the cosine fit was considered when F was more than $F_{0.05}$ ($F > F_{0.05}$); Acrophase time was considered when the mRNA level at a given time-point was within 95% confidence bounds of the acrophase; The value R^2 quantified goodness of fit; Phase shift was the time lag between the acrophase time in the control condition and that in resetting days; ns, no significant 24-h rhythm.

Figure Captions

Liver

○ - Control ● - Resetting

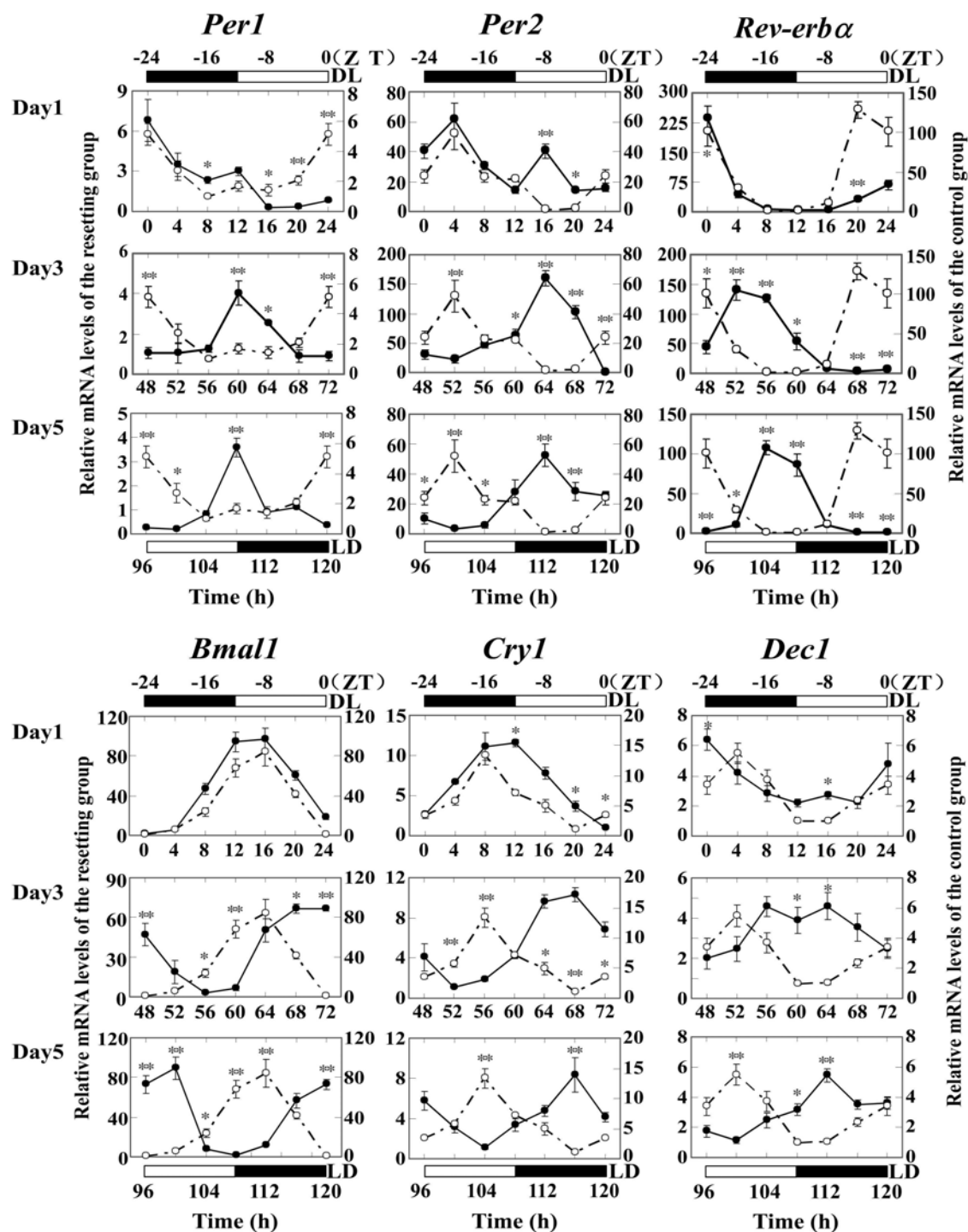


Figure 1. Resetting processes of clock genes in the liver after the reversal of feeding schedule and light-dark cycle via a 24-h light period transition

Expression profiles of clock genes were examined in the liver of rats in the control

condition and days after the feeding and LD reversal. Filled circles / solid lines represent data for the resetting group, and open circles / dashed lines indicate data for the control group. The values on the left side of each panel show the corresponding mRNA levels in the resetting group, while the values on the right side of each panel show the corresponding mRNA levels in the control group (the ZT24 value represents a replotting of the ZT0 value). The black bars on the top of each column represent the duration of the dark phase in the control group; whereas, the black bars on the bottom of each column represent the duration of the dark phase in the resetting group. The quantitative representations of multiple results are expressed as values relative to the minimum value of the control group in LD condition. Each value represents the mean \pm SEM of four animals ($*p < 0.05$; $**p < 0.01$).

Figure 2. Resetting processes of clock genes in the heart after the reversal of feeding schedule and light-dark cycle via a 24-h light period transition

Expression profiles of clock genes were examined in the heart of rats in the control condition and days after the feeding and LD reversal. The explanation of each symbol is as described in Figure 1. The quantitative representations of multiple results are expressed as values relative to the minimum value of the control group in LD condition. Each value represents the mean \pm SEM of four animals ($*p < 0.05$; $**p < 0.01$).