Preventive effect of L-Carnitine on the disorder of lipid metabolism and circadian clock of mice subjected to chronic jet-lag

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Short title: Effect of L-Carnitine on prolonged circadian disruption

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Summary

Circadian clock plays an essential role in orchestrating daily physiology, and its disruption can evoke metabolic diseases such as obesity. L-Carnitine can reduce blood lipid levels, and ameliorate fatty liver through regulating lipid metabolism. However, whether L-Carnitine administration may affect the disturbance of lipid metabolism and circadian rhythm of mice induced by prolonged circadian disruption is still unknown. Herein, we investigated the effects of L-Carnitine on conditions of circadian clock and lipid metabolism through a chronic jet-lag mice model which was developed by reversing12h light/12h dark cycle every 4 days for a continuous 12 weeks. Results showed that L-Carnitine administration significantly decreased levels of serum glutamic-oxaloacetic transaminase (GOT) and triglycerides (TG), which were remarkably elevated by chronic jet-lag. More importantly, quantitative real-time polymerase chain reaction (qRT-PCR) analysis indicated that L-carnitine supplementation would effectively counteract the negative alterations in gene expression which related to lipid metabolism (*Srebp1*, *Acaca*, *Fasn,* and *Scd1*), metabolic regulator (*mTOR*) and circadian rhythm (*Bmal1*, *Per1*, *Cry1* and *Dec1*) in the liver of mice subjected to the chronic jet-lag. As a conclusion, L-Carnitine was partly effective in preventing the disruption of circadian clock and lipid metabolic disorders induced by the chronic jet-lag.

Key words

Chronic jet-lag • Circadian clock • L-Carnitine • Lipid metabolism • Mice

Introduction

The approximately 24 hour light-dark (LD) cycle drives cyclic changes in the living environments for most organisms on earth from cyanobacteria to human beings. The mammalian circadian clock is composed of a master pacemaker and peripheral oscillators and takes an essential role in orchestrating daily physiology, including sleep/wake, body temperature, feeding, hormone secretion, and metabolism. Systemic circadian regulation is accomplished by the central oscillator in the superachiasmatic nucleus (SCN) of the anterior hypothalamus. While, the peripheral clocks present in most vital organs, such as heart, liver, adipose tissue, and muscle (Albrecht 2012, Partch *et al.* 2014, Rey and Reddy 2013, van Alphen and Allada 2014). The molecular mechanism for oscillation in SCN and peripheral tissues is generated by interlocked negative transcriptional/translational feedback loops (Brown *et al.* 2012, Dibner *et al.* 2010), which are formed by several core clock genes including *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry1*and *Cry2*) and modulated by CLOCK-BMAL1 proteins. Orphan nuclear receptors REV-ERB and ROR families are also reported as the feedback regulative targets of CLOCK-BMAL1 (Bugge *et al.* 2012, Kohsaka *et al.* 2007).

Circadian clocks in our bodies provide time cues for activities and the synchronization of the metabolic reactions (Green *et al.* 2008, Sahar and Sassone-Corsi 2012). Proper function of circadian clock is of great importance in regulating physiological process. Until now, several external stimuli, such as overtime work, night eating, sleep disruption, as well as frequent shift/jet lag (Haus and Smolensky 2006, Leloup and Goldbeter 2013), and chronic shift in LD cycles have been reported to influence the function of Circadian clock (Oike *et al.* 2015). Further, the disruption can lead to internal desynchronization between the master clock and

other peripheral oscillators, and increase the risk of many diseases, including obesity and other metabolic syndromes (Marcheva *et al.* 2010, Sahar and Sassone-Corsi 2009, Turek *et al.* 2005). Chronic jet lag leads to the dysregulation of leptin in adipose and central leptin resistance in wild-type mice, resulting in a high and arrhythmic serum leptin level over a 24 hr period (Kettner *et al.* 2015), which may be associated with an obvious increase in body weight and fat composition (Wu *et al.* 2015). Chronic jet lag also disrupts the endogenous adipose clock, and abolishes the circadian rhythm of BMAL1 binding to leptin and Per1 promoters (Kettner *et al.* 2015). These findings demonstrate that chronic jet lag might be closely associated with lipid metabolism and endogenous adipose clock in mice.

As known, L-carnitine is involved in long-chain fatty acids transporting from cytosol to the mitochondria matrix (Marcovina *et al.* 2013), which is required for facilitating lipid metabolism and reducing the storage of long-chain fatty acids in adipose. In our previous study, we found that L-carnitine supplementation could prevent irregular feeding-induced lipid metabolism disorder (Wu *et al.* 2015). However, whether L-Carnitine may affect the disorder of circadian rhythm and lipid metabolism of mice subjected to prolonged circadian disruption is still no reported. In the present study, we developed an experimental chronic jet-lag mice model by reversing12h light/12h dark cycle every 4 days for a continuous 12 weeks to investigate the effects of L-Carnitine on the lipid metabolism and circadian clock. The results demonstrated that L-Carnitine supplementation prevented the impairment of the serum markers, and effectively counteracted the negative alterations in the expression of lipid metabolic genes and clock genes in mice.

Materials and Methods

Materials

L-Carnitine (Aladdin Chemistry Co. Ltd, Shanghai, China) was mixed with normal commercial diet at 0.5% w/w (L-Carnitine containing diet). A feeding of this diet (12.5 mg L-Carnitine/mouse/day) was equivalent to a dosage of about 400 mg of L-Carnitine per kg of mouse weight each day. To ensure each mouse could consume the entire 12.5 mg of L-Carnitine every day, mice were fed with 1g L-Carnitine containing diet (20% of total food intake) at first 2 h, and then fed with 80% of normal commercial diet after they had eaten up the L-Carnitine containing diet.

Animals and experimental design

Male C57BL/6 mice (6-weeks old) were used in this research. The mice were housed in temperature-controlled (22±1 °C) quarters on a LD cycle 12:12, and provided water *ad libitum* and food only in the dark period. The onset of light was defined as zeitgeber time 0 (ZT0) and the onset of darkness at ZT12.

After 7 days of acclimatization, mice were randomly divided into three groups of control (Con), jet-lag (JL) and jet-lag+carnitine (JL+C). The experimental design was shown in Fig. S1. Mice of JL+C group were fed with L-Carnitine containing diet, and other mice were fed with normal commercial diet (5.0 g/mouse/day). In Con group, mice were kept under the normal LD condition. In JL and JL+C groups, mice were subjected to a reversal of LD cycle every 4 days for a continuous 12 weeks. Then after fasting for 12 h, the mice in all three groups were sacrificed at ZT0 and ZT12 under the LD cycle.

The tissues of all mice were collected, frozen immediately in liquid nitrogen, and kept at −80 °C for RNA extraction. Blood was also collected and centrifuged at 6000 *g* for 5 min at 4 °C, then stored at −40 °C. Every effort was made to minimize animal suffering and the number of mice required for each experiment. All experiments were

performed according to institutional guidelines, and the study was approved by the Research Committee of Zhejiang University of Technology.

Biochemical analysis

Plasma levels of glutamic-pyruvic transaminase (GPT) and glutamic-oxaloacetic transaminase (GOT) were measured by auto-biochemical analysis system (Achtection c8000; Abbott, North Chicago, Illinois, USA). The levels of triglycerides (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) were analyzed using commercial kits (Whitman Biotech, Nanjing, China).

Quantitative real-time PCR

The cDNA templates were isolated from the mouse livers as previously described (Xie *et al.* 2014). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on an Eppendrorf MasterCycler ep RealPlex4 (Wesseling-Berzdorf, Germany), with the SYBR ExScript PCR Kit (TOYOBO, Tokyo, Japan). The primer sequences of the selected genes used in the present study were shown in Table S1. The relative expression levels were calculated by $2^{-\Delta\Delta CT}$ method according to the previous description (Schmittgen and Livak 2008, Wu *et al.* 2008). The results were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase *(GAPDH).*

Western blotting

The proteins were isolated from hepatic samples, and their concentrations were measured using BCA Protein Assay Kit (Beyotime Institute of Biotechnology, China). The lysate was mixed with 5×SDS sample buffer and boiled for 10 min. Lysate samples were separated on 6% and 12% SDS–polyacrylamide gels, and transferred to a PVDF membrane. The blots were blocked with 5% milk blocking solution for 2 h at room temperature and then incubated overnight with antibodies against PER1 (1:1000;

Abcam, USA), mTOR (mammalian rapamycin), Phospho-mTOR (1:1000; Cell Signaling Technology, USA), and β-actin (1:1000; Beyotime Institute of Biotechnology). HRP-conjugated anti-rabbit IgG antibody (1:1000; Beyotime Institute of Biotechnology) was used as the secondary antibody. The blots were visualized by ECL Western Blotting Detection Reagents (Beyotime Institute of Biotechnology) and the images were performed by GEL imaging system (Bio-Rad, USA). The quantification of proteins was analyzed by the software Quantity One (Bio-Rad, USA).

Data Analysis

Data are presented as mean ± SEM. The values for mRNA levels are presented as relative values in all experiments. Data were checked for normality and homogeneity of variance using the Kolmogorov-Smirnov one-sample test and Levene′s tests, respectively, before conducting statistical comparison. As the assumptions were met, the data were subjected to one-way analysis of variance (ANOVA).

Results

Effects of L-Carnitine on serum markers of mice subjected to chronic jet-lag

To investigate the effects of L-Carnitine on serum markers, the activity levels of GPT, GOT, TG, TC and HDL-C at ZT12 were measured. As shown in Fig.1, the activity levels of serum GPT and GOT were significantly higher in the JL group (Table S2, $p < 0.05$) compared to those in the Con group. However, when supplemented with L-Carnitine, the serum GPT and GOT activities were decreased as compared with those in the JL group, and they did not exhibit obvious differences (Table S2, $p > 0.05$) to the Con group (Fig.1A-B). Moreover, a higher serum TG and

TG/HDL-C ratio in the JL group were observed compared to the Con group (Table S2, $p < 0.05$) (Fig. 1C, E), whereas there were no differences in the concentrations of serum TC and TC/HDL-C ratio among three groups ($p > 0.05$; Fig. 1D, 1F).

Effects of L-Carnitine on hepatic mRNA levels of genes involved in adipogenesis

To test the effects of L-Carnitine on the lipid metabolism in mice subjected to chronic jet-lag, the mRNA levels of peroxisome proliferator activated receptor γ (*PPARγ*), sterol regulatory element binding protein 1 (*Srebp1*), Acetyl-CoA carboxylase (*Acaca*), fatty acid synthase (*Fasn*), stearoyl-CoA desaturase1 (*Scd1*) and 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (*Hmgcr*) in the liver at ZT12 were analyzed. Among them, the mRNA levels of *Acaca*, *Fasn* and *Scd1* were significantly increased by the chronic jet-lag treatment as compared with respective ones of the control, which were reduced significantly (Table S3, $p < 0.01$) by the supplementation with L-Carnitine. In addition, the supplementation with L-Carnitine also dramatically decreased the *Srebp1* mRNA level (Table S3, $p < 0.01$) compared with that of both the Con and JL groups (Fig. 2B-E). No significant change was observed in the mRNA levels of *PPARγ* and *Hmgcr* among the three groups at ZT12 (Table S3, $p > 0.05$; Fig. 2A, F).

Effect of L-Carnitine on the expression of lipolytic genes and glycometabolism-related genes (Gck and Pck1) in the liver

To explore the role of L-Carnitine supplementation in the expression of lipolytic genes, the mRNA levels of *PPARα*, carnitine palmitoyl transferase 1 (*Cpt1*), *Cpt2*, carnitine/acylcarnitine translocase (*Slc25a20*) in the liver at ZT12 were examined (Fig.3).The chronic jet-lag treatment increased the mRNA level of *PPARα* and significant reduction in the mRNA levels of *Cpt2* and *Slc25a20* when compared with respective ones of the control, which were down-regulated partially (Fig. 3A and 3B)

or completely (Fig. 3D) by the L-Carnitine supplementation. L-Carnitine supplementation had no effect on the expression of *Cpt1*which was induced by the chronic jet-lag (Fig. 3C).

Hepatic PPARγ is involved in provision of glycerol-3-phosphate (G3P) which is required for TG synthesis and storage (Nakamura *et al.* 2014). To investigate the effect of L-Carnitine on the PPARγ targeting genes, the mRNA levels of glucokinase (*Gck*) and phosphoenolpyruvate carboxykinase 1 (*Pck1*) were measured (Fig. 3E, F). The chronic jet-lagtreatment elevated the mRNA level of *Gck* at both ZT0 and ZT12, with a significant difference at ZT12 as compared with that of the control (Table S4, $p < 0.01$). These increased changes were effectively lowered by L-Carnitine supplementation. Similar results were also observed for *Pck1* mRNA levels. Briefly, the mice exhibited a significant up-regulation of *Pck1* expression (Table S4, $p < 0.05$) in the JL group compared with those in the Con group at ZT0 and ZT12, while they exhibited a significant down-regulation of *Pck1* expression (Table S4, $p < 0.01$) in the JL+C group compared with those in the JL group at ZT12.

Effect of L-Carnitine on mTOR activity in the liver

mTOR, a metabolic regulator, promotes light-evoked protein translation (e.g. PERIOD protein). It is also involved in lipid synthesis and energy metabolism (Cao *et al.* 2010, Laplante and Sabatini 2012). To investigate the effects of L-Carnitine on mTOR activity, mTOR mRNA level, total mTOR and phosphorylated mTOR (P-mTOR) protein levels were examined. As shown in Fig.4A, the mRNA level of *mTOR* in the JL group was significantly higher than that in the Con group at both ZT0 and ZT12 (Table S5, $p < 0.05$), but was decreased by supplementation of L-Carnitine ($p < 0.05$). Protein level of mTOR was significantly lower in the JL+C group as

compared to that in the JL group at ZT12 (Table S5, $p < 0.05$). However, P-mTOR protein levels were similar among the three groups at both ZT0 and ZT12 (Fig.4B).

Effect of L-Carnitine on hepatic mRNA and protein levels of circadian clock markers

To test whether L-Carnitine plays a role in the regulation of circadian clock, we analyzed its effects on liver clock gene expression in mice subjected to a prolonged circadian disruption. As shown in the Fig 5A, the expression level of circadian clock genes (*Bmal1*, *Per1*, *Cry1* and *Dec1*) were increased significantly (Table S6, $p < 0.05$) in the JL group compared with those in the Con group at ZT0 and ZT12. L-Carnitine supplementation attenuated the impact on the expression of clock genes caused by the prolonged circadian disruption, and led to a significant decrease (Table S6, $p < 0.05$) in their mRNA levels as compared to that of the JL group, except the expression of *Per1* at ZT0 (Table S6, $p > 0.05$). Moreover, the protein level of PER1 was increased markedly (Table S6, $p < 0.05$) in the JL group compared with that in the Con group at ZT0 and ZT12, which was decreased significantly (Table S6, $p < 0.01$) in the JL+C group compared with that in the JL group.

Discussion

L-Carnitine, a nutritional element, is supplemented in foods for healthy humans. It lowers lipid levels in the blood, and reduces high fat-induced obesity (Kim *et al.* 2007, Liu *et al.* 2015). It also ameliorates fatty liver through the regulation of carnitine-dependent lipid metabolism, and prevents lipid metabolism disorder caused by irregular feeding (Wu *et al.* 2015), which is intertwined with circadian clock. Moderate dietary supplementation of L-Carnitinehas a prominent effect on peripheral organs, and affects the body′s daily rhythms including performance, core body temperature, and alertness in both human and rodent studies (Asher and

Sassone-Corsi 2015, Damiola *et al.* 2000, Liu *et al.* 2015). The findings suggest that L-Carnitine might delay the onset of degenerative syndromes caused by irregular feeding. In the present study, we found that L-Carnitine had preventive effects on lipid metabolism disorder and circadian clock dysfunction in mice exposed to the prolonged reversal of 12 h photo-schedule.

Our findings showed a significant disturbance in the lipid metabolism in mice subjected to the chronic jet-lag, being consistent with previous descriptions (Biggi *et al.* 2008, De Bacquer *et al.* 2009). The enhanced levels of hepatic enzymes of GPT and GOT by the chronic jet-lag could be indicative of liver injuries, possibly leading to hepatic maladaptation, which might be responsible for the increased serum levels of TG and TG/HDL cholesterol ratio. It is worth to point out that the supplementation of L-Carnitine could effectively prevent such lipid metabolism disturbance and liver injuries, suggesting that L-Carnitine might be used to protect the possible hepatic maladaptation from the frequent shift-workers.

The significant up-regulated expression of lipogenic genes *Acaca*, *Fasn* and *Scd1* was produced by the chronic jet-lag, which was similar with the previous observations (Barclay *et al.* 2012). Such increased expression could be suppressed by L-carntine administration, suggesting L-carntine might be beneficial for hepatic steatosis, hyperlipidemia, and atherosclerosis (Li *et al.* 2011, Lima-Cabello *et al.* 2011). The expression of *Hmgcr*, a susceptible gene responsible for cholesterol de novo biosynthesis, was not altered by exposing to the prolonged reversal of the photo-schedule, which was in line with the unaltered serum total cholesterol.

The carnitine palmitoyl transferase (CPT) system is mainly regulating fatty acid β-oxidation, and L-Carnitine transports long-chain fatty acid into the mitochondrial matrix (Priore *et al.* 2012). The frequent alteration of the photo-schedule had an

impact on the expression of clock genes, and reduced mRNA level of *Cpt1*, *Cpt2*, *Slc25a20* in this system, suggesting that it might inhibit fatty acid oxidation, which was also observed by Li et al. (Li *et al.* 2014). However, the decreased mRNA levels of *Cpt2* and *Slc25a20* could be completely counteracted by L-Carnitine supplementation, demonstrating that supplementation of L-Carnitine was of benefit for long-chain fatty acids transporting into mitochondria, and thus might improve fatty acid metabolism in hepatic tissue (Longo *et al.* 2006).

Mice subjected to the chronic jet-lag exhibited significantly higher expression of *Gck* and *Pck1* genes, the proteins of which may contribute to the activation of PPARγ and the increase of the synthesis of TG via glycerol 3 phosphate (Nakamura *et al.* 2014). The increased expression of these two genes was reduced by L-Carnitine supplementation, suggesting that it might decrease TG level through inhibiting the expression of genes related with glycometabolism. In addition, the mTOR protein, a serine/threonine kinase, belongs to plosphoinositide 3-kinase (PI3K) related kinase family (Logan *et al.* 2012). It has been reported that the binding of insulin to the cell surface receptor activates PI3K, which positively up-regulates de novo lipogenesis by promoting glucose uptake, the expression of genes involved in lipid biosynthesis, and the deposition of excess carbohydrates to be stored as TG in hepatic tissue (Laplante and Sabatini 2009, Manning and Cantley 2007). These help explain our results that the increased TG synthesis was accompanied by the elevated expression of mTOR in the liver of mice. Thus, the decrease of mRNA and protein levels of mTOR by L-Carnitine administration, demonstrated that L-Carnitine attenuated the disruption of lipid metabolism of mice subjected to the chronic jet-lag, which was similar with the previous description (Kettner *et al.* 2015), through regulating mTOR pathways.

Mice subjected to the frequent shift of LD cycle exhibited significantly higher

expression of hepatic clock genes (*Bmal1*, *Per1*, *Cry1* and *Dec1*), which are indirectly regulated by light as previously described (Iwamoto *et al.* 2014, Reppert and Weaver 2002). Giving the close relationship of the PER protein expression with mTOR pathways, the PER1 and mTOR protein expressions were examined. The increased expression of both proteins by the chronic jet-lag supports the idea that light-evoked mTOR signaling may be required to augment PER protein expression (Cao and Obrietan 2010). Interestingly, increased protein levels of PER1 and the increased mRNA levels of *Bmal1*, *Per1*, *Cry1* and *Dec1* were clearly down-regulated by L-Carnitine containing diet, suggesting that L-Carnitine might play a positive role in the circadian adaptation, and might also be pivotal in stabilizing endogenous clock through evoking many pathways, such as mTOR. This hypothesis could be partially supported by accumulated studies on the essential role of circadian clock genes in interacting with several crucial metabolic factors for regulation of metabolic processes (Bugge *et al.* 2012, Cao and Obrietan 2010, Takeda *et al.* 2014).

In summary, the present study demonstrated that the exposure to the chronic jet-lag could lead to physiological maladaption, the disturbance of hepatic lipid metabolism and circadian clock in mice. L-Carnitine supplementation could effectively counteract the negative alterations in the serum marker levels, and the expression of the genes regulating the lipid metabolism and hepatic clock rhythm. Our findings might provide the essential data toward elucidating the complicate relationship among L-Carnitine，lipid metabolism and hepatic circadian clock.

Conflict of Interest

There is no conflict of interest.

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Figure Legends

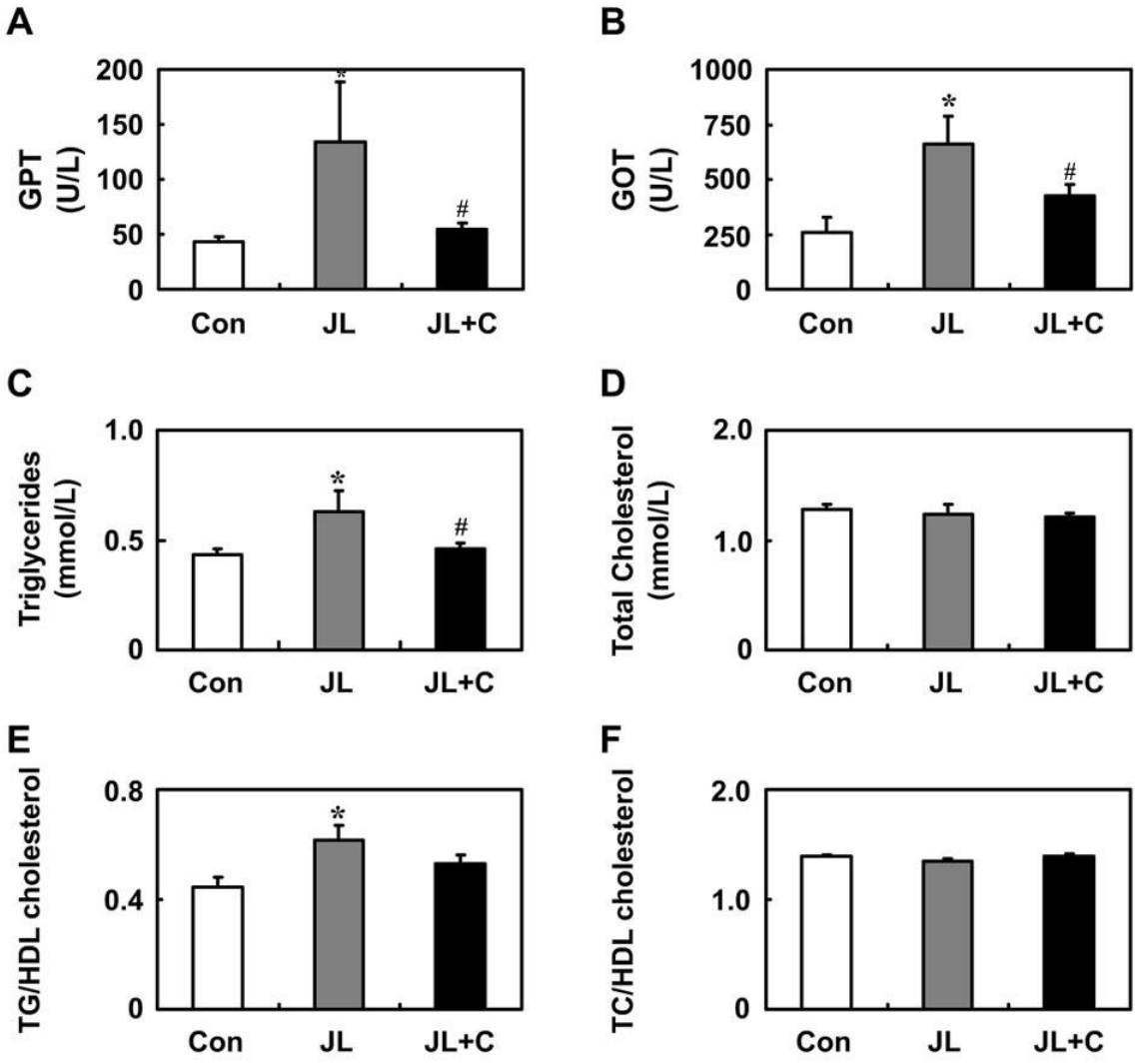

Figure 1. Effect of L-Carnitine on serum parameters

After one week of acclimatization, mice were randomly divided into three groups of control (Con), jet-lag (JL) and jet-lag+carnitine (JL+C). Mice of JL+C group were fed with L-Carnitine containing diet, and other mice were fed with normal commercial diet (5.0 g/mouse/day), during their active state (dark phase). In the Con group, mice were kept under LD conditions. In the JL and JL+C groups, mice were subjected to a reversal of LD cycle every 4 days for a continuous 12 weeks. At the end of experiments, the serum concentration of GPT (A), GOT (B), TG (C), TC (D), TG/HDL-C (E) and TC/ HDL-C (F) were analyzed. Values are expressed as mean ±

SEM (n=5). *$p < 0.05$ compared with the control group; $^{\#}p < 0.05$ compared with the JL group. (A, B) liver enzymes (GPT, GOT): indicative of liver injury; (C-F) functional disturbance (TG, TC, TG/HDL-C, TC/HDL-C): compromised hepatic metabolism (adipogenesis).

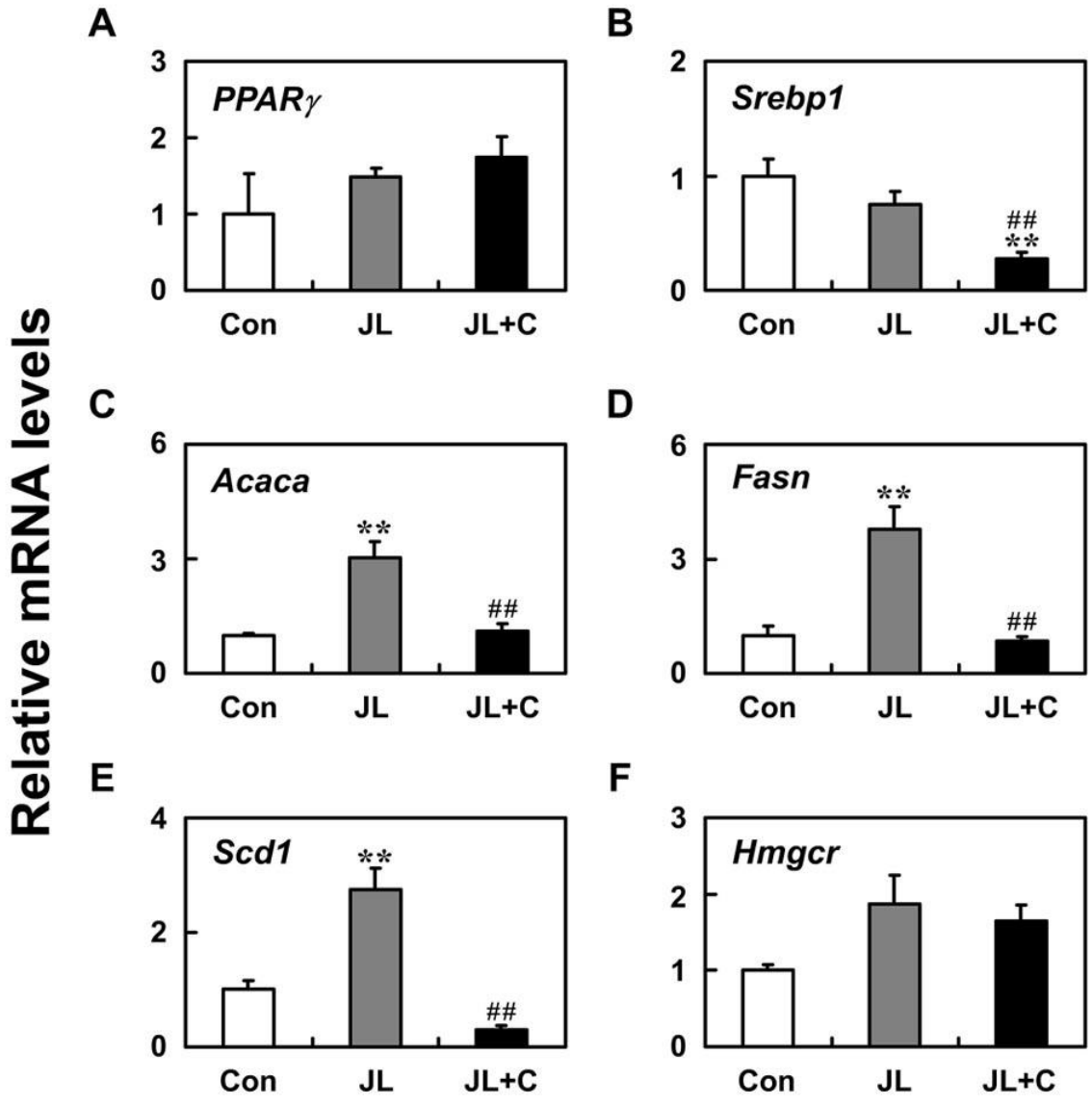

Figure 2. Effect of L-Carnitine on hepatic mRNA levels of genes involved in adipogenesis

The mRNA levels of genes related to adipogenesise were determined by qRT-PCR in the livers of Con, JL and JL+C mice. The mRNA level was normalized using *GAPDH*.

Each value represents the mean ± SEM (n=5). *p < 0.05 compared with the control group; #p< 0.05 compared with the JL group.

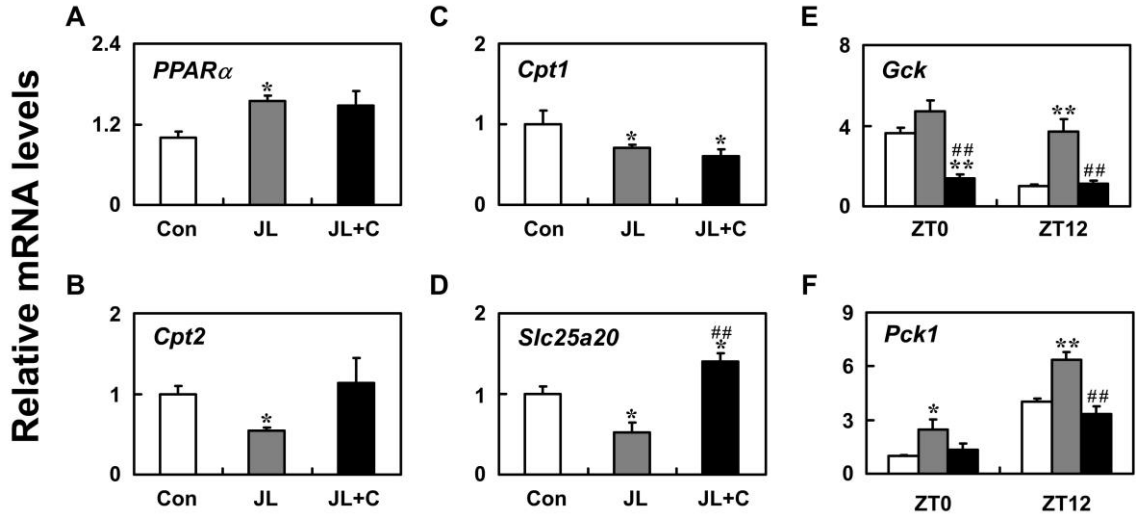

Figure 3. Effect of L-Carnitine on hepatic mRNA levels of lipolytic genes and glycometabolism-related genes (*Gck and Pck1*)

qRT-PCR was used to determine the mRNA levels of lipolytic genes and glycometabolism-related (*Gck* and *Pck1*) genes in the liver of Con, JL and JL+C mice. The mRNA amount was normalized to the expression of *GAPDH* mRNA. Values are expressed as mean ± SEM (n=5). *p< 0.05 compared with the control group; #p< 0.05 compared with the JL group. Values are expressed as mean ± SEM (n=5).

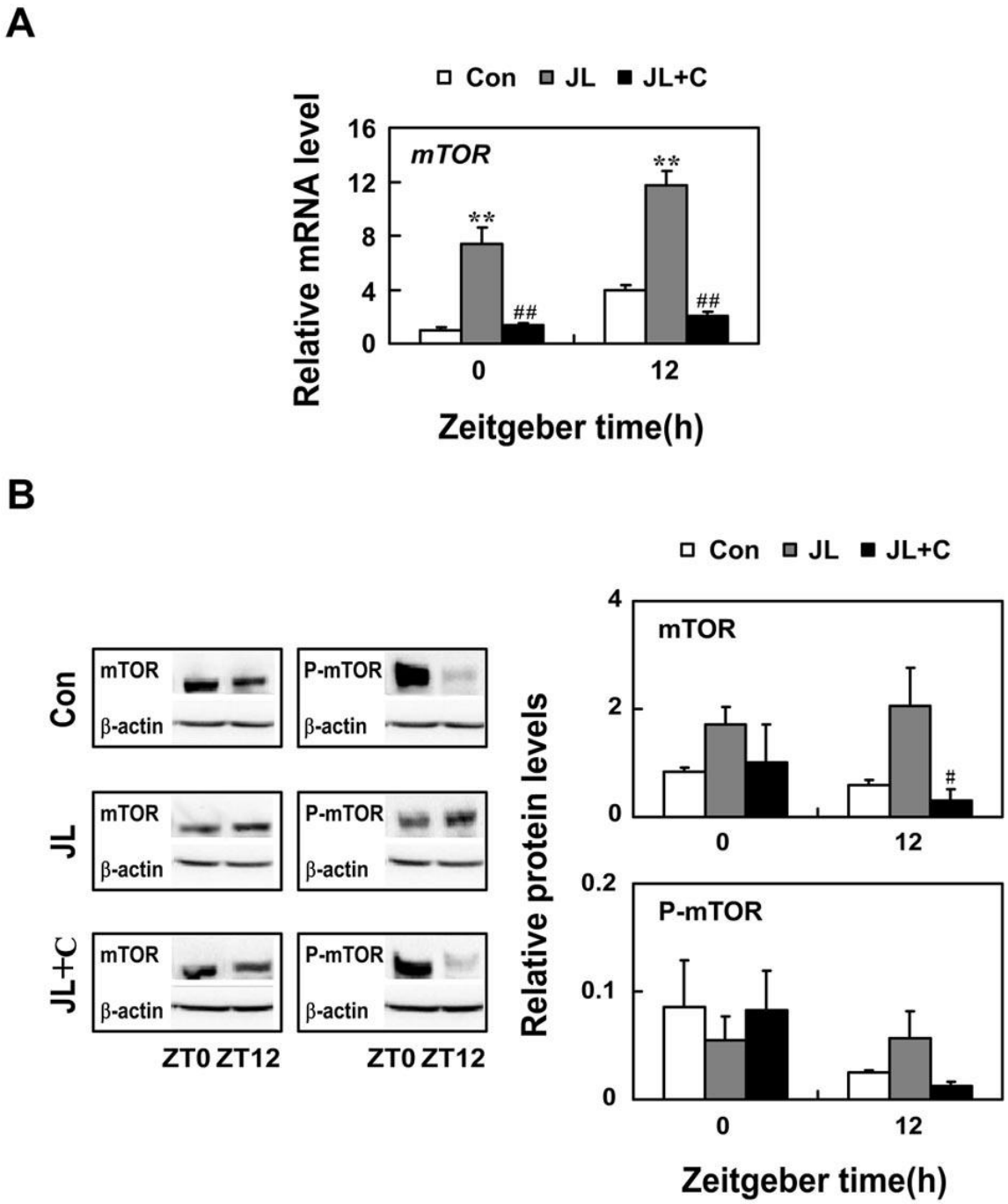

Figure 4. Effect of L-Carnitine on mTOR activity

(A) qRT-PCR was performed to examine the mRNA level of *mTOR* gene in the liver. The results were normalized to the expression level of the *GAPDH* gene. Each value represents the mean ± SEM (n=5).

(B) Western blot was performed to examine the protein levels of total mTOR and

phosphorylated mTOR in the liver of Con, JL and JL+C mice. β-actin was used for signal normalization. The protein content was quantified by densitometric analysis of blots. Each value represents the mean ± SEM (n=3).

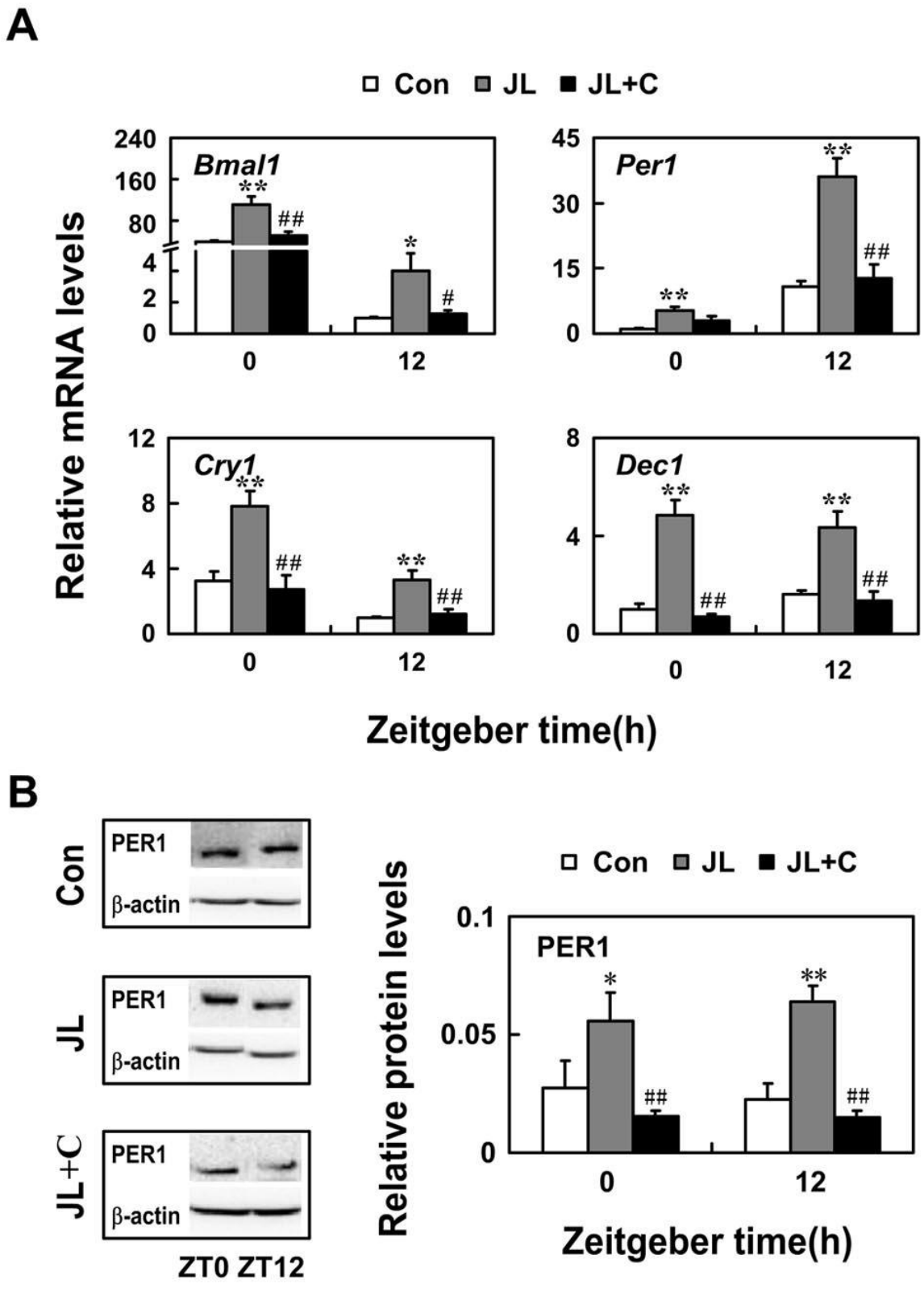

Figure 5. Effect of L-Carnitine on circadian clock mRNA and protein levels

(A) The mRNA levels of clock genes were determined by qRT-PCR in the liver of Con, JL and JL+C mice. The results were normalized to the expression level of the

GAPDH gene. Values are expressed as mean ± SEM (n=5). *$p < 0.05$ compared with the control group; #$p< 0.05$ compared with the JL group. Values are expressed as mean ± SEM (n=5).

(B) Western blot was performed to test PER1 expression in the liver of Con, JL and JL+C mice. The PER1 protein content was quantified by densitometric analysis of blots. β-actin antibody served as loading control. Values are expressed the mean ± SEM (n=3).

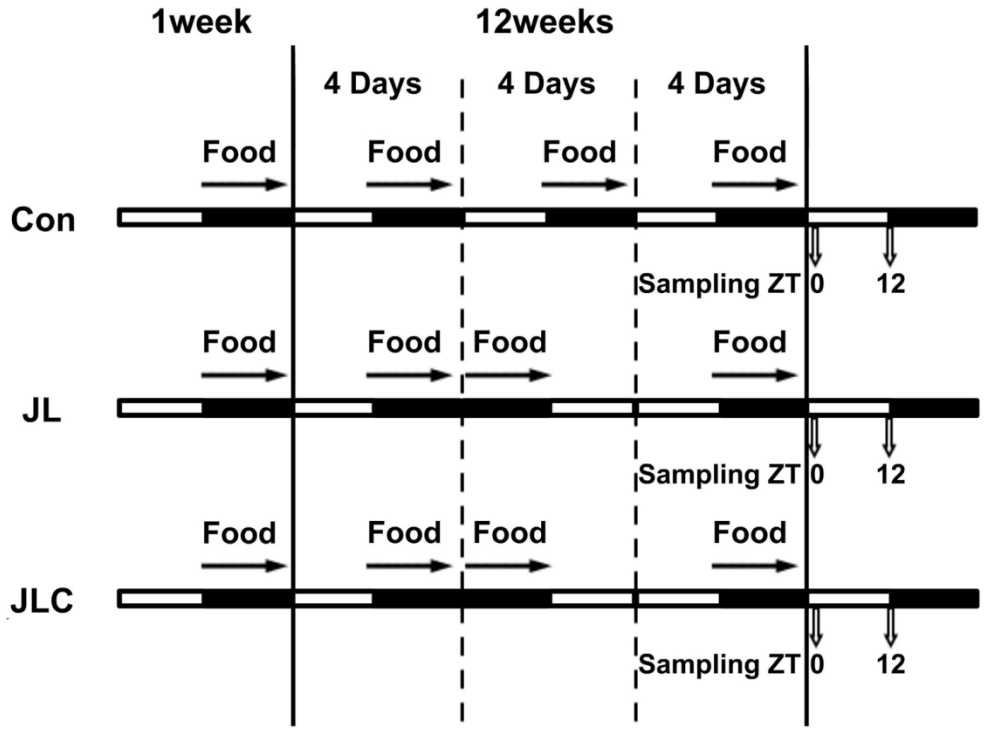

Fig. S1 Experimental Schedule

After seven days of acclimatization, mice were randomly divided into three groups of the Control group (Con), the Jet lag group (JL) and the Jet lag+Carnitine group (JL+C). The schedule of feeding and chronic jet-lag is shown in Fig.S1. Mice of the JL+C group were fed with L-carnitine containing diet, and other mice were fed with

normal commercial diet (5.0 g/mouse/day), corresponding feeding time during active state (dark phase). In the Con group, mice were kept under light-dark (LD) cycle 12:12 conditions. In the JL and JL+C groups, mice were subjected to a reversal of LD cycle every 4 days for a continuous 12 weeks.

Table 1

Primer sequences used for qRT-PCR amplification

Gene	Accession number		Primer sequence 5' to 3'
GAPDH	**NM_008084.2**	Forward,	GACCTCAACTACATGGTCTACA
		Reverse,	ACTCCACGACATACTCAGCAC
PPARγ	**NM_001127330.1**	Forward,	CCAACTTCGGAACTCAGCTCTG
		Reverse,	AACCTGATGGCATTGTGAGACA
Srebp1	**NM_011480.3**	Forward,	GGCACTAAGTGCCCTCAACCT
		Reverse,	GCCACATAGATCTCTGCCAGTGT
Acaca	**NM_133360.2**	Forward,	TAACAGAATCGACACTGGCTGGCT
		Reverse,	ATGCTGTTCCTCAGGCTCACATCT
Fasn	**NM_007988.3**	Forward,	GCAGCAAGTGTCCACCAACAA
		Reverse,	CTCATCGGAGCGCAGGATAGA
Scd1	**NM_009127.4**	Forward,	GATAGAGCAAGTCCCCGTTG
		Reverse,	CCTGCATTAACCCCCTTCAC
Hmgcr	**NM_008255.2**	Forward,	CAGCTTACAGAGCCAATGATGGAG
		Reverse,	AGCCCATAAATGATTCAGTCACCAA
PPARα	**NM_011144.6**	Forward,	CCTCAGGGTACCACTACGGAGT
		Reverse,	GCCGAATAGTTCGCCGAA
Cpt1	**NM_031559**	Forward,	CACTGGCCGAATGTCAAG
		Reverse,	TGCAAACATCCAGCCGTG
Cpt2	**NM_009949**	Forward,	GACAGCCAGTTCAGGAAGACAG
		Reverse,	TATTCTGTTTATCCTGAGCGAGC
Slc25a20	**NM_020520**	Forward,	GAGAGGGCATCACAGGGCT
		Reverse,	CTTCCCCAGACCAAACCCA
Gck	**NM_010292**	Forward,	TGGACAAGCATCAGATGAAACA
		Reverse,	TGGACAAGCATCAGATGAAACA
Pck1	**NM_011044.2**	Forward,	GTGTTTGTAGGAGCAGCCATGAGA
		Reverse,	GCCAGGTATTTGCCGAAGTTGTAG
Bmal1	**NM_007489.4**	Forward,	AAGTGCAACAGGCCTTCAGT
		Reverse,	GGTGGCCAGCTTTTCAAATA
Per1	**NM_011065.4**	Forward,	CCCAGCTTTACCTGCAGAAG
		Reverse,	AGCTGGGGCAGTTTCCTATT
Cry1	**NM_007771.3**	Forward,	AGCTGGGGCAGTTTCCTATT
		Reverse,	CATCTCGTTCCTTCCCAAAA
Dec1	**NM_011498.4**	Forward,	GACCGGATTAACGAGTGCAT
		Reverse,	TCAATGCTTTCACGTGCTTC
mTOR	**NM_020009.2**	Forward,	GTCCGCCTTCACAGATACCC
		Reverse,	TGATGTCAAGTACACGGGGC

qRT-PCR: quantitative real-time PCR

Table 2 Interaction between groups on serum markers of mice subjected to chronic jet-lag

Groups	GPT		GOT		TG		TC		TG/HDL-C		TC/HDL-C	
	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value
JL comparing to Con	4.482	0.031	9.325	0.022	3.441	0.0499	0.256	0.631	7.803	0.013	3.064	0.155
JL+C comparing to Con	2.715	0.148	3.492	0.104	0.389	0.773	1.897	0.211	3.392	0.103	0.078	0.790
JL+C comparing to JL	2.628	0.149	3.520	0.120	2.548	0.084	0.068	0.803	2.218	0.187	3.005	0.134

GPT: glutamic-pyruvic transaminase; GOT: glutamic-oxaloacetic transaminase; TG: triglyceride; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol.

Table 3 Interaction between groups on hepatic mRNA levels of genes involved in adipogenesis

Groups	*PPARγ*		*Srebp1*		*Acaca*		*Fasn*		*Scd1*		*Hmgcr*	
	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value
JL comparing to Con	1.071	0.348	1.891	0.228	17.587	0.0041	14.868	0.0062	15.201	0.006	3.837	0.107
JL+C comparing to Con	1.881	0.229	28.59	0.0017	0.255	0.629	0.457	0.521	18.957	0.082	7.058	0.045
JL+C comparing to JL	0.805	0.404	15.24	0.0059	16.994	0.033	22.414	0.0015	40.146	0.0001	0.211	0.665

PPARγ: peroxisome proliferator activated receptor γ; Srebp1: sterol regulatory element binding protein 1; Acaca: Acetyl-CoA carboxylase; Fasn: fatty acid synthase; Scd1: stearoyl-CoA desaturase1; Hmgcr: 3-hydroxy-3-methyl-glutaryl coenzyme A reductase.

Table 4 Interaction between groups on the expression of lipolytic genes and glycometabolism-related genes (Gck and Pck1) in the liver

	PPARα		*Cpt1*		*Gck (ZT0, ZT12)*		*Cpt2*		*Slc25a20*		*Pc*
	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value	F valu
on	21.063	0.0059	4.699	0.0487	3.582,14.305	0.117,0.0069	22.996	0.0049	7.763	0.0317	8.83,28
Con	3.248	0.1314	5.27	0.017	23.795,0.309	0.0018,0.5954	0.124	0.7393	7.168	0.044	1.225,2.
JL	0.0081	0.7877	1.558	0.4188	39.067,16.443	0.0004,0.0037	3.552	0.1084	28.223	0.0011	2.884,25

PPARα: peroxisome proliferator activated receptor α; Cpt1: carnitine palmitoyl transferase 1; Gck: glucokinase; Slc25a20: carnitine/acylcarnitine translocase; Pck1: phosphoenolpyruvate carboxykinase 1.

Table 5 Interaction between groups on mTOR activity in the liver

Groups	*mTOR* (mRNA)				mTOR (protein)				P-mTOR (protein)			
	ZT0		ZT12		ZT0		ZT12		ZT0		ZT12	
	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value
JL comparing to Con	34.559	0.0006	35.102	0.0006	7.329	0.0537	4.16	0.0533	0.487	0.5356	1.605	0.176
JL+C comparing to Con	1.733	0.2245	17.549	0.0041	0.065	0.812	1.604	0.6582	0.003	0.9628	0.994	0.5513
JL+C comparing to JL	31.187	0.0008	71.226	0.0001	0.843	0.414	10.551	0.0286	0.403	0.5601	3.136	0.0736

mTOR: metabolic regulator; P-mTOR: phosphorylated mTOR.

Table 6-1 Interaction between groups on mTOR activity in the liver

Groups	*Bmal1*				*Per1*				*Cry1*			
	ZT0		ZT12		ZT0		ZT12		ZT0		ZT12	
	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value
JL comparing to Con	20.063	0.0021	5.32	0.0212	21.39	0.0017	27.484	0.0012	16.564	0.0036	11.707	0.0111
JL+C comparing to Con	2.244	0.1725	1.36	0.8104	4.096	0.0776	0.287	0.6096	0.226	0.6469	0.385	0.5545
JL+C comparing to JL	11.628	0.0092	4.306	0.0318	2.969	0.1232	20.215	0.0025	15.254	0.0045	9.609	0.0147

Table 6-2 Interaction between groups on mTOR activity in the liver

Groups	*Dec1*				PER1 (protein)			
	ZT0		ZT12		ZT0		ZT12	
	F value	p value	F value	p value	F value	p value	F value	p value
JL comparing to Con	33.42	0.0004	13.743	0.0076	4.941	0.00367	25.715	0.0071
JL+C comparing to Con	1.38	0.2738	0.301	0.6005	2.597	0.3113	1.868	0.2435
JL+C comparing to JL	43.975	0.0002	16.118	0.0039	11.154	0.0091	44.937	0.0026